Postharvest Biology and Technology of Fruits, Vegetables, and Flowers

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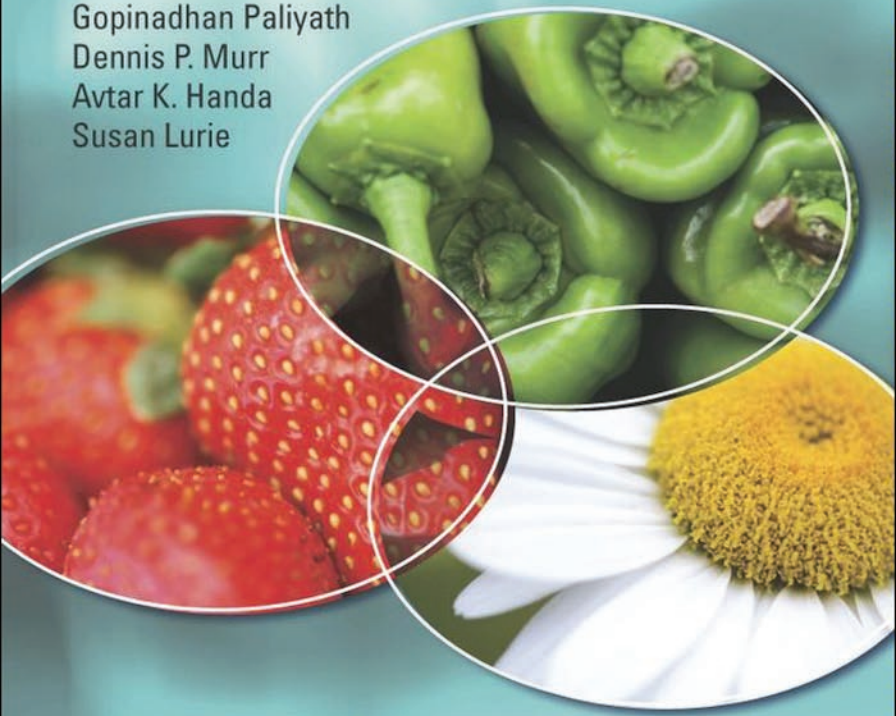

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Preface

Postharvest practices for the preservation of fruits, vegetables, and flowers are perhaps one of the oldest in human history. With the understanding of the molecular processes that occur during plant senescence, this discipline has developed unique features of its own. Traditionally, postharvest science is considered as an applied science focusing on the physiological aspects of enhancement of shelf life and preservation of quality of horticultural produce. However, in the past two decades, biochemical and molecular biological aspects have been extensively used for analyzing postharvest issues. The present work is a compilation of some of the advances in basic aspects of postharvest science.

Postharvest issues are common around the world. The extent of the loss of horticultural produce after harvest can vary in different countries. In those parts of the world where the methods of agricultural production and storage employ advanced technology, postharvest losses may be minimal, and most of it occurs during the transit of produce from the production site to the destination along the consumer chain. The losses can range from 10 to 20% by volume. In tropics where the production practices are basic and based on day-to-day demand, the postharvest losses can be as high as 50% or over. It is surprising and a bit disturbing to see that fruits are considered as luxury items in some parts of the world. In an era where we consider the consumption of fruits and vegetables as a means of health promotion, postharvest science gets a new meaning. Therefore, to compile the key aspects of postharvest science through international authors has been helpful in addressing some of these issues.

A common theme in most chapters is to link the biochemical and molecular aspects relevant to that topic with postharvest implications. As well, several novel advances in the area have been brought out. The chapters have been organized in such a way that the physiological aspects are linked to the postharvest aspects. The chapters on biochemistry of fruits, followed by flowers, give an overall picture of metabolic processes in horticultural produce. The role of ethylene receptors, 1-MCP technology to block ethylene receptor action changes to cell wall and membrane structure that occur during ripening, biosynthesis of quality components, technologies for shelf life enhancement and quality preservation and quality determination, and nutritional aspects, etc., have been dealt with in the following chapters. Overall, the book covers several topics that are not normally dealt within the books dealing with postharvest science at present. An example will be the chapter on membrane deterioration. The importance of membrane preservation and thus maintenance of membrane compartmentalization is a topic that has been dealt with for over 20 years. It is

difficult to find a book that deals with this topic at present. In addition, we have also described the technology of membrane preservation through the inhibition of phospholipase D, the key enzyme that initiates membrane phospholipid degradation. Phospholipase D inhibition technology using hexanal shows great promise and may serve as a widespread technology for the preservation of organically grown horticultural produce.

While preparing this book, we have given adequate consideration to present the most advanced information on all topics. In a constantly evolving field, this is always not easy; however, the contents are intended for students in the area of horticulture and postharvest science as well as scientists and other professionals. We believe that the contents will enrich the knowledge and provide a fresh angle to several issues in postharvest science. As well, the contributions by internationally reputed experts in the area provide great depth to the individual topics.

The editorial team wishes to thank all the contributors for their great effort and taking time in completing the chapters in an excellent fashion and making this effort into a success. The editorial team also wishes to thank the whole-hearted support, encouragement, and professionalism shown by the publishing team at Wiley-Blackwell during the preparation of the book and bringing it into a successful production.

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Postharvest Biology and Technology of Fruits, Vegetables, and Flowers

Chapter 1

Postharvest Biology and Technology: An International Perspective

Gopinadhan Paliyath, Dennis P. Murr, Avtar K. Handa, and Susan Lurie

1.1 Importance of fruits, vegetables, and flowers in world economy

The production of fruits, vegetables, and flowers has been an important sector in the total world agricultural output. Although an accurate economic contribution from these segments around the world is difficult to obtain because of the reporting systems, it is estimated that the contribution from these segments could be over US$600 billion. The globalization of trade and free trade agreements have tremendously increased the transaction and transport of these commodities across nations and continents. The weather patterns in the Northern Hemisphere are not suited for the routine production of tropical produce, and therefore fruits such as banana, pineapple, mango, citrus fruits, and tomato are exported to countries in the Northern Hemisphere. The production of flowers in North America has severely been affected by imports from South and Central America. Thus, fruits, vegetables, and flowers are produced and transported from South and Central America and Mexico to the United States and Canada; from Europe to Asia; from India to the Middle East; from Israel and Egypt to Europe; from China to the Pacific region; and from Australia, New Zealand, and South Africa to countries around the world. Canada alone, with a population of nearly 30 million, imports these commodities valued in excess of US$3 billion. The cost of production is a factor that has positively influenced such trades. Countries where labor costs are low can effectively compete with lower-priced produce. These factors have influenced the local economies and agricultural production practices in several countries.

Several international and governmental agencies have kept track of fruits and vegetables available for consumption on a per capita basis. In the United States, the amount of fresh and processed fruits available for consumption increased from 263 lb per person per year in 1976 to 277 lb per person per year in 2003, registering a 0.2% growth (Cook, 2004). Similarly, the amount of fresh and processed vegetables available for consumption increased from 622 lb per person per year in 1976 to 712 lb per person per year in 2003, registering a 0.5% growth (Cook, 2004). Statistics Canada (Food Statistics, 2007) estimates that total fruits available for consumption increased from 117 kg per capita in 1993 to 133 kg per capita in 2004. The per capita availability of vegetables decreased from 187 kg in 1993 to 178 kg in 2004.

1.2 Importance of fruits and vegetables as food

Human evolution was potentially linked initially to the consumption of naturally available fruits and vegetables, which later might have resulted in the selection of preferred plants and varieties for agriculture. The cultivation of grapes and its processing into wine is a classic example of the use of fruits. Fruits and vegetables are also used in traditional medical systems such as Ayurveda. Fruits and vegetables are major sources of several essential nutrients that include vitamins A and C and folic acid. In addition, fruits and vegetables are rich in antioxidants such as carotenoids, polyphenols, and anthocyanins that help combat free radicals produced within the body and the excess production of which has been related to the development of cardiovascular diseases, Alzheimer's, macular degeneration, and cancers. Fruits and vegetables are integral components of food in all societies; however, in some parts of the world, this is limited due to agricultural collapse or sociopolitical conflicts. Fruits are considered as high-value items and not readily accessible to economically challenged segments of population around the world. With the results from a number of epidemiological studies spanning several countries and continents and population groups showing the relation between increased fruit and vegetable consumption and a reduced risk of developing maladies such as cardiovascular and cerebrovascular diseases, cancer, neurodegenerative diseases such as Alzheimer's, and macular degeneration. A very positive attitude toward fruit and vegetable consumption has emerged recently, especially in advanced countries. In Canada, fruits and vegetables form an important segment of the food guide. Daily servings of 5 or more comprising a variety of fruits, vegetables, and their processed products have been recommended both by the US and Canadian federal agencies. Flowers are also of great importance as food, examples being cauliflower and broccoli.

A new food guide was released by United States Department of Agriculture (USDA) in 2005 (USDA, 2005; Center for Nutrition Policy and Promotion) providing clear recommendations on fruit and vegetable intake. The recommendations for vegetables include the intake of 2.5 cups equivalent (250 mL per cup) per day or more, comprising dark green vegetables such as broccoli and spinach; orange vegetables such as carrots and sweet potatoes; and dry beans and lentils. A recent Canadian publication (www.healthcanada.gc.ca/foodguide, Health Canada Publication 4651, 2007) shows recommendations ranging from 4 to 6 servings (a serving is 125 mL or $\frac{1}{2}$ cup) for children, from 7 to 8 servings for teens, and from 7 to 10 servings for adults. So far as the fruits are concerned, the recommendations are to eat a minimum of 2 cups equivalent every day comprising a variety of fruits and processed products, excluding juice. High sugar content in fruit juices may not be suitable for many health-compromised individuals. Whole fruit blends without added sugar may be an option in such cases. Detailed information is also available on the fruit and vegetable consumption habits of Americans (Lin et al., 2004). In a 2003 estimate (Guthrie et al., 2005), it was found that typical American diet falls severely short of the daily recommendations having fruit consumption equivalent to 1.4 servings per day (less than half of the recommended 4 servings or 2 cups). Vegetable consumption is relatively higher with 3.7 servings per day (below the recommended 5 servings or 2.5 cups per day). However, promotions at several levels (media, organizations, schools, fast food chains, etc.) are resulting in an increased understanding of the importance of fruit and vegetable consumption.

Table 1.1 Production of selected fruits (million metric tons) in major geographical areas of the world

Commodity	Africa	Asia	China	India	Europe	North and Central America	USSR	World
Apple	2.02	34.36	25.01	1.47	13.03	5.15	4.42	63.48
Apricot	0.39	1.19	<0.1	<0.1	0.77	<0.1	0.32	2.82
Banana	7.44	38.86	6.39	16.82	<0.1	8.95	—	72.46
Blueberry	—	—	—	—	0.26	0.20	<0.1	2.41
Cantaloupes and melons	1.45	21.48	15.14	6.45	3.16	2.48	<0.1	28.32
Cherry	<0.01	0.61	<0.1	<0.1	0.67	0.26	0.26	1.86
Citrus fruits	11.62	37.07	16.02	4.75	10.24	19.55	<0.1	105.4
Dates	2.38	4.52	—	—	—	—	—	6.92
Grapes	3.73	15.66	5.70	1.20	29.43	6.96	2.55	66.53
Mango	2.71	21.18	3.67	10.80	—	2.48	—	27.96
Peach	0.77	7.78	6.03	0.15	4.26	1.62	0.21	15.67
Pear	0.62	13.38	11.62	0.2	3.33	0.82	0.39	19.51
Plum	2.29	5.46	4.63	<0.1	2.76	3.76	0.56	9.84
Pineapple	2.61	8.39	1.46	1.30	—	2.22	—	15.86
Strawberry	—	—	—	—	1.11	1.15	0.27	3.52
Tomato	14.63	60.21	31.64	7.60	19.40	17.25	6.39	124.75
Watermelon	3.77	80.41	69.31	0.25	3.09	3.09	3.36	95.29

FAO Statistics (2005).

1.3 Fruit, vegetable, and flower production around the world

Every part of the world produces fruits and vegetables mostly according to the needs of domestic consumption and export. Table 1.1 summarizes the production statistics of certain selected fruits. Apples, bananas, citrus fruits, grapes, tomatoes, and watermelons are the largest fruit commodities produced. Asia is the largest producer of fruits, with China being the primary producer in the whole world. Although the populations of China and India are nearly the same, fruit production in India is considerably lower than that in China. This may be due to the differences in food habits between the two countries, with the Chinese population consuming a lot more fresh fruits and products. On a per capita basis, Israel produces more fruits than any other country in the world. Europe and North and Central America are also the major players in fruit production. Chile is the major producer of fruits in South America. Overall, the world produces greater than 600 million metric tons of fruits (FAO Statistics, 2005).

World vegetable production also follows similar trends as in fruits (Table 1.2), Asia being the largest producer of vegetables. Again, China is the powerhouse of vegetable production, fresh vegetables being a major component of daily diet. Europe and the Americas also contribute significantly to vegetable production. Altogether, the world vegetable production exceeds 650 million metric tons (FAO Statistics, 2005).

In contrast to fruit and vegetable production, the bulk of flower production is concentrated in specific regions of the world. The production and marketing of flowers and potted ornamentals are intricately linked across the world. A floral bouquet purchased in a local supermarket may be made with miniature carnations from Israel, chrysanthemums from Columbia, boxwood from Oregon, and statice from California. Worldwide trade in floriculture products was estimated to exceed US$7.9 billion in 2001 and was made up of cut

Table 1.2 Production of selected vegetables (million metric tons) in major geographical areas of the world

Commodity	Africa	Asia	China	India	Europe	North and Central America	USSR	World
Asparagus	—	5.98	5.90	—	0.25	0.17	—	6.65
Avocados	0.38	0.45	<0.1	—	<0.1	1.5	—	3.22
Cabbage	1.77	50.16	34.10	6.00	6.32	2.99	7.59	69.48
Cauliflower	0.30	13.04	7.38	4.80	2.11	0.64	—	16.36
Carrot	1.13	10.71	8.39	0.35	5.16	2.38	3.19	23.90
Green chillies and peppers	2.20	16.31	12.53	<0.1	2.72	3.05	0.20	24.98
Cucumbers and gherkins	1.05	33.01	26.56	0.12	4.62	2.11	3.12	41.74
Eggplant	1.41	28.22	17.03	8.2	0.6	0.1	0.1	30.51
Lettuce	0.27	13.21	11.00	0.79	3.15	5.37	—	22.38
Onions (dry)	5.12	35.30	19.04	5.50	5.57	4.35	3.56	57.59
Potato	15.39	125.71	73.77	25.00	65.16	26.53	73.72	321.97
Sweet potato	11.47	114.99	107.67	0.90	—	1.47	—	129.88

FAO Statistics (2005).

flowers (50%), plants (41%), bulbs (9%), and cut foliage (9%). Over 70% of the production of floriculture crops is concentrated in seven countries: the Netherlands, Columbia, Italy, Belgium, Denmark, the United States, and Ecuador. The Netherlands is the flower capital of the world, with over 50% of world trade (2000 estimate, www.agf.gov.bc.ca/ornamentals; British Columbia, 2003) in floriculture products that include produce grown within the Netherlands (farm gate value of US$5.4 billion), as well as floriculture products that are imported and marketed again to various destinations. Columbia was the second largest exporter with an estimated trade at 7.5%, and the rest of the flower-producing countries having export components of 2–3% each. The major flower markets (70%) include Germany, the United States, Britain, France, the Netherlands, and Japan. In the United States and Canada, the production has shifted from flowers such as carnations, chrysanthemums, and roses to speciality cut flowers such as gerbera, *Lizianthus*, snapdragons, and *Alstroemeria* that are relatively more difficult to grow and transport. Also, the cultivation of potted ornamentals is on the rise. According to USDA (2002) estimates, there were over 8,000 ha in floriculture production with a wholesale value estimated at US$4.88 billion. A similar estimate by Statistics Canada (2002) provides a value of Canadian $1.4 billion for Canadian floriculture products.

Flower production has become an important agricultural segment in some Asiatic countries. Again, on a per capita basis, Israel dominates the scene with an estimated 2,750 ha in 2005 (S. Meir, personal communication) and an estimated value of US$250 million. China had nearly 60,000 ha in flower and foliage crop production and India had nearly 35,000 ha according to 1994 estimates, which has expanded recently to include greenhouse production and marketing to the Middle East. Australia and New Zealand are the principal suppliers of floriculture products to Pacific Rim countries and Japan.

1.4 Postharvest loss of fruits, vegetables, and flowers

By virtue of their physiological properties, most fruits, vegetables, and flowers are highly perishable commodities. Postharvest losses can occur at any point in the production and

marketing chain, and may range anywhere from >10% in advanced countries to >50% in tropical areas and where storage facilities are limited. China, the largest fruit and vegetable producing country, experiences postharvest losses in the range of 20–25%. Since a large proportion of fruits and vegetables produced are immediately consumed, the loss from long-term storage is considerably reduced. As well, processing can also reduce postharvest losses. China is the largest producer of apple juice concentrate, and this reduces the necessity for large storage facilities. Moreover, such facilities are not suitable since the sizes of farms are rather small. Cold storage facilities may be available for 10–15% of the fruit crops in China. Cooling and transportation facilities are also being developed in China. In India, the problem of storage for pulses is reduced by letting the fresh fruits mature and dry. Several fruits from the cucumber family are ripened, after which they can be stored for months without specialized storage facilities. In countries such as India and China where the weather is tropical and subtropical, fruits and vegetables characteristic to these climates are produced. As well, the production of many of such commodities is seasonal, and this reduces the necessity of long-term storage. Thus, it is common to have peaks of availability for fruits such as apple, orange, pear, banana, mango, and guava spread throughout the year. Even then, postharvest loss of 50% or greater is common as the storage facilities at the local vendors are limited and good quality products are hard to find (e.g., apple).

1.5 Strategies to improve quality

A recent understanding among the growers on the effect of growing conditions on the quality of produce has brought a welcome change in the attitude toward the goals of production. Simply producing a commodity in large amounts need not assure the optimum postharvest quality of the produce. In general, the quality of a produce cannot be enhanced through adopting postharvest storage technologies. The quality of a produce is determined by the growing conditions, nutritional regimes, and the genetic potential of the particular variety. Thus, increased attention is being given to these attributes. Several novel postharvest technologies developed in recent years have the potential to maintain the high quality of produce during subsequent storage at optimal conditions. These include active modified atmosphere and dynamic controlled atmosphere. In addition, there is growing concern about food safety, which is also being addressed in the postharvest area. Growers in Europe and those in other countries who export to Europe are now required to implement the standards of Good Agricultural Practice. These standards are to ensure the safety and quality of fresh produce and require accountability and traceability of produce entering the European market. This means that the farmer must keep a record of the irrigation, fertilization, and pest management treatments that he applies; the packinghouse, exporter, and the shipper must record the treatments given at the packinghouse and storage conditions of the produce along the distribution chain. It also requires safety and cleanliness conditions for the workers in the farms and packinghouse. The producer and exporter must be examined by an external evaluator to ensure that Good Agricultural Practice is being implemented.

The necessity for traceability has led to the development of barcodes and radiofrequency identification stickers that can trace a commodity from “farm to fork.” Some farms and packinghouses now have barcodes on each container in the orchard that is read as it enters the packinghouse and is weighed and checked. Radiofrequency identification systems have

been given to both orchard pickers and packinghouse workers. As fruit is packed, the packer prints a label that includes a barcode, grower, count, date and time, pack house and variety details, and packer. This enables fruit to be traced back to the orchard and row from which it was harvested and to know all the steps it took along the way to the consumer.

In the area of produce quality, packinghouses have units of quality assessment, where samples of the produce entering the packinghouse as well as along the packing line are examined for quality. Recent innovations are instruments that can determine various aspects of quality nondestructively. On many packinghouse lines, currently fruit is automatically graded for color and blemishes by online cameras that photograph the fruit and send it to the proper sorting line. This is combined with sorting for weight or size by automatic weighing cups that send different sizes to different lines. Newer technologies include near-infrared spectrometers that can examine internal quality, particularly soluble solids or sugars, as well as acoustic instruments that can measure firmness. These are now being supplied in new packinghouse lines, and in addition, hand-held instruments are in development for use in orchards to determine picking date.

Biotechnological approaches are also useful for enhancing the shelf life and quality of fruits, vegetables, and flowers, but the public acceptance of this technology is limited. Several information sites describing the optimal storage procedures have also increased the importance and understanding of postharvest storage (e.g., Sydney Postharvest Laboratory, www.postharvest.com.au/; www.usda.org).

1.6 Future prospects

Demographic and socioeconomic factors are the major influencing trends that affect fruit and vegetable consumption in the United States (Guthrie et al., 2005). Rising income and education levels as well as increasing average age of the population may also influence this trend. In the United States, an increase in Hispanic population is predicted to increase the consumption of tomato and its products. By the year 2020, per capita intake of potatoes and french fries is expected to drop by 8–9%. The consumption of lettuce (+5.1%), tomatoes (+1.3%), and other vegetables (+3.6%) is expected to increase during the same period. Among fruits, the consumption of grapes (+5.1%), apples (+7.8%), citrus fruits (+7.4%), and other fruits (+7%) is anticipated to increase by the year 2020. However, an increased tendency to eat outside the home may tend to reduce the fruit intake marginally, as such a habit tends to promote the intake of lettuce and potato products marginally. In Canada, the United States, and Europe, a large increase in Asian population is anticipated to increase the consumption of fruits and vegetables native to Asia. Another important trend in fruit and vegetable production is organic farming. It is generally believed that fruits and vegetables grown organically are more nutritious and the production of organic fruits and vegetables is increasing yearly. Farmers' markets and pick-your-own operations have increased the diversity of fruit and vegetable production and marketing. Sustainable and environment-friendly production methods are increasingly being applied in most countries. Addition of fruit components to processed food (cereals, yogurt, etc.) has become a common trend in the processing industry. Thus, several socioeconomic factors are driving the importance of fruit and vegetable consumption in a positive direction, and this in turn will influence the patterns of production, storage, and distribution of fruits and vegetables.

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Chapter 2

Common Fruits, Vegetables, Flowers, and Their Quality Characteristics

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2.1 Introduction

Fruits and vegetables as well as their processed products have become mainstream human dietary choices in recent days, primarily because of several epidemiological studies showing various health benefits associated with the consumption of fruits, vegetables, and their processed products. Fruits and vegetables share several common structural and nutritional properties and also characteristic differences due to differences in their biochemical composition. Fruits, in general, are attractive organs for vectors involved in seed dispersal, and thus have evolved features such as enhanced color, attractive flavor, and taste. Consequently, the developmental and biochemical processes within a fruit are programmed to achieve this goal. The term *vegetables* is more or less arbitrary, comprising products such as leaves, petioles, stems, roots, tubers, and fruits of cucurbits (e.g., gourds, melons, squash, and pumpkin) and Solanaceae members (e.g., tomato and eggplant). Morphologically, fruits develop from the ovary, the seed-bearing structure in plants. The developmental processes in fruits are influenced by fertilization, and the hormonal changes induced in the ovary leads to gene expression and biochemical changes resulting in the characteristic fruit that may vary in ontogeny, form, structure, and quality. Fruits originate from different parts of the ovary. Pome fruits such as apple and pear develop from the thalamus of the flower. In drupe fruits such as cherries, peaches, plums, and apricots, the ovary wall (mesocarp) develops into the fruit enclosing a single seed. Berry fruits, such as tomato and grape, possess the seeds embedded in a jellylike pectinaceous matrix, with the ovary wall developing into the flesh of the fruit. Citrus fruits belong to the class known as hesperidium, where the ovary wall develops as a protective structure surrounding the juice-filled locules that are the edible part of the fruit. In strawberry, the seeds are located outside the fruit, and it is the receptacle of the ovary (central portion) that develops into the edible part. Most vegetables are leaves, petioles, or stems containing chlorophyll, or roots, tubers, or fruits that predominantly contain storage components such as starch. Examples include potato and eggplant (Solanaceae), gourds (Cucurbitaceae), several types of yams (Dioscoreaceae and Araceae), vegetables of leaf and flower origin (cabbage, broccoli, cauliflower—Cruciferae), and unripe fruits of leguminous plants such as peas and beans (Leguminosae). The nutritional and food qualities of fruits and vegetables arise as a result of the accumulation of components derived from the intricate biochemical pathways (Kays, 1997).

2.2 Developmental characteristics of fruits and vegetables

Fruit ripening is characterized by several marked physiological and biochemical changes resulting in the coordinated development of complex characteristics. Following pollination and fertilization, the fruit develops in size leading to the ripening process, which results in the development of ideal organoleptic characters such as taste, color, and aroma that are important quality-determining features. Fruits that are used as vegetables are harvested early prior to their ripening. The physiological process of ripening occurs rapidly when the fruit is mature, and beyond a certain stage, harvested fruits undergo rapid deterioration in quality. Ideally, fruits are harvested at an optimal physiological stage or maturity characteristic to the type of fruit, after which appropriate storage procedures can be adopted for preserving the shelf life and quality of the fruits. Fruits do not ripen fully showing the appropriate quality characteristics if picked at a young stage before the attainment of physiological maturity. Citrus fruits are allowed to fully ripen before they are harvested. Avocado fruits do not ripen if left on the tree and start ripening only after harvest. Irrespective of the nature of the produce whether it is fruits, vegetables, or flowers, various technologies such as cold storage, controlled atmosphere storage, and inhibition of hormone and enzyme action are adopted to slow down the metabolic processes to provide an optimal quality produce for marketing and consumption. Advances in the biochemistry and molecular biology of the fruit ripening process have enabled the development of biotechnological strategies for the preservation of postharvest shelf life and quality of fruits, vegetables, and flowers.

Several metabolic changes are initiated after the harvest of fruits and vegetables. In the case of vegetables, harvesting induces stress responses through reduced availability of water and nutrients, wounding, and exposure to shelf life enhancing storage methods such as cooling. In most cases, these changes help the produce to enhance the shelf life. In the case of fruits, an increase in the biosynthesis of the gaseous hormone ethylene serves as the physiological signal for the initiation of the ripening process. In general, all plant tissues produce a low, basal, level of ethylene. During the ripening process, some fruits evolve large amounts of ethylene, sometimes referred to as an autocatalytic increase in ethylene production, which occurs in conjunction with an increase in respiration referred to as the respiratory climacteric. Fruits are generally classified into climacteric or nonclimacteric types on the basis of the pattern of ethylene production and responsiveness to externally added ethylene. The climacteric fruits characteristically show a marked enhancement in ethylene production and respiration, as noticeable by the evolution of carbon dioxide. By contrast, the nonclimacteric fruits emit a considerably reduced level of ethylene. (For a list of fruits showing climacteric or nonclimacteric pattern of ripening, see Kays, 1997, General Reading.) In climacteric fruits such as apple, pear, banana, tomato, and avocado, ethylene evolution can reach 30–500 ppm/(kg h) (parts per million, microliter per liter), whereas in nonclimacteric fruits such as orange, lemon, strawberry, and pineapple, ethylene levels usually range from 0.1 to 0.5 ppm/(kg h) during ripening. Climacteric fruits respond to external ethylene treatment by an early induction of the respiratory climacteric and accelerated ripening in a concentration-dependent manner. Nonclimacteric fruits, on the other hand, show increased respiration in response to increased levels of ethylene concentration without showing acceleration in the time required for ripening. Vegetables produce very low amounts of ethylene most of them with less than 0.1 μL/(kg h), with slightly higher levels as

in cassava (1.7 μL/(kg h)), breadfruit (1.2 μL/(kg h)), and cucumber (0.6 μL/(kg h)) when measured at 20–25°C.

After the initiation of ripening or harvest, several biochemical changes occur in fruits and vegetables. As some of these changes such as the development of color, flavor, and sweet taste are desirable for fruits, any sort of quality changes are ideally not desired in vegetables. Thus, strategies for the preservation of shelf life and quality in fruits and vegetables could be entirely different. It is important to know the biochemical differences between fruits and vegetables and several biochemical pathways that operate in these tissues to develop ideal conditions of storage for the preservation of shelf life and quality.

2.3 Biochemical parameters of quality

There are two major aspects that define the quality of a produce: the first being the inherent biochemical characteristics that provide the color, flavor, texture, and taste to the produce and the second being the consumer perception. The application of postharvest technologies tends to maximize these quality characteristics, though application of some technologies may not provide the optimal quality produce for the consumer. During ripening, activation of several metabolic pathways occurs, often leading to ideal changes in the biochemical composition of fruits. The stage of development in a fruit determines its biochemical composition and the quality-defining parameters. Color is perhaps the first parameter that attracts a consumer to a produce. Hence, fruits that show enhanced yellow-orange-red hue are preferred by the consumer. The composition of anthocyanins and carotenoids in a fruit will determine its color quality characteristics. Consumers also associate the depth of color with the taste, though this is influenced by practical experiences. In general, fruits that are bright red are also sweet. Some of the exceptions include sour cherries and red currants. Brightly colored fruits also tend to possess the ideal texture. Flavor is also an important component to the quality perception, and the degree of ripeness determines the level and types of flavor components such as esters and terpenoids emitted from the fruit. Aroma is derived from several types of compounds that include monoterpenes (as in lime, orange), ester volatiles (ethyl, methyl butyrate in apple, isoamyl acetate in banana), simple organic acids such as citric and malic acids (citrus fruits, apple), and small-chain aldehydes such as hexenal and hexanal (cucumber). In fruits such as mango, pineapple, strawberry, and grape, the ripening process is associated with the conversion of stored organic acids and starch into sugars, and enhanced evolution of flavor components. The presence of off-flavors resulting from the presence of certain aldehydes (e.g., acetaldehyde) may negatively impact quality perception, whereas other aldehydes such as hexanal tend to enhance the green flavor and consumer preference of vegetables. The evolution of sulfur volatiles in crucifer vegetables (e.g., broccoli and cabbage) and *Allium* vegetables (onion, garlic) is characteristic to their quality. In a similar way, the evolution of essential oils in Lamiaceae members (mint, oregano, rosemary, etc.) also attracts consumers. Fruits and vegetables contain a large percentage of water, which can often exceed 95% by fresh weight. Texture and the degree of softness are determined by the amount of water contained in the produce and the ability to retain that water during postharvest storage. The degradation of cell wall components and the cell membrane negatively affects the rigidity of the tissue in fruits providing the softness that consumers prefer (degradation of stored starch in banana), though excessive degradation of these components reduces the shelf life of the fruits drastically. Most vegetables

are preserved to maximize the high textural integrity, and loss of water from vegetables negatively affects their quality. The consumers are increasingly becoming aware of the disease-preventive and health-restoring roles of fruits and vegetables, because of which they are classified as functional foods. Many quality-determining components are also regarded as important functional food ingredients (nutraceuticals) that include soluble and insoluble fibers, color components such as anthocyanins and carotenoids, several polyphenolic components, and sulfur-containing components in crucifer and *Allium* vegetables. Fruits in general contain large amounts of fibrous materials such as cellulose and pectin. The breakdown of these large polymers into smaller water-soluble components during ripening leads to fruit softening as observed during the breakdown of pectin in tomato and cellulose in avocado. Secondary plant metabolites are major ingredients of fruits. Anthocyanins are the major color components in grapes, blueberries, apples, and plums; carotenoids, specifically lycopene and carotene, are the major color components in tomatoes, and these components provide the health benefits to consumers through their antioxidant property and ability to influence metabolic processes within the human body. Fruits are also rich in vitamin C, which is a strong antioxidant. Vegetables such as asparagus are rich in glutathione, another component in the antioxidant defense system. Lipid content is quite low in fruits; however, fruits such as avocado and olives store large amounts of triacylglycerols (oils). The amounts of proteins are usually low in most fruits. Several aspects that influence fruit and vegetable quality are discussed by Shewfelt and Bruckner (2000).

It is interesting to note that a majority of edible fruits and vegetables tend to group in certain families. For instance, some of the edible fruit-dominated families include Annonaceae, Rosaceace, Myrtaceae, Rutaceae, Oxalidaceae, and Anacardiaceae among the dicots. Bananas and plantains are the major monocot fruits (Musaceae). The major dicot vegetable families include Fabaceae, Solanaceae, Cruciferae, Cucurbitaceae, Compositae, Umbelliferae, Lamiaceae, and Dioscoreaceae. Monocot families such as the Liliaceae and Araceae are rich in vegetables. The following are some specific characteristics of fruits and vegetables.

2.4 Fruits

2.4.1 Rosaceae

The family Rosaceae dominates the scene with a variety of fruits having distinct physiological and nutritional characteristics. Rosaceous members are cultivated primarily in the subtropic and temperate regions. Unripened fruits are tart and the ripened fruits are in general sweet with varying degrees of acid content, which imparts the sour sweet taste to the fruits. The fruits are also rich in polyphenolic components such as anthocyanins, vitamin C, and soluble as well as insoluble fiber, which make them extremely important for consumption. The fruits of the Rosaceae include blackberry, raspberry (*Rubus* sp.), strawberry (*Fragaria ananassa*), plum (*Prunus* sp.; European plum—*Prunus domestica*; Japanese plum—*Prunus salicina*; American plum—*Prunus Americana,* etc.), prune (*P. domestica*), nectarine and peach (*Prunus persica*), cherry (sweet cherry—*Prunus avium*; sour cherry—*Prunus cerasus*), apple (*Malus domestica*), pear (*Pyrus communis*), quince (*Cydonia oblonga*), loquat (*Eriobotrya japonica*), etc. Apples have a very long shelf life when stored under appropriate controlled atmosphere conditions. Pears are usually stored below 0°C for a time period

without controlled atmosphere, after which they can be brought to room temperature (RT) for ripening. Most of the soft rosaceous fruits such as the berries, cherries, plums, prunes, and peaches have a short storage life and are highly perishable.

2.4.2 Rutaceae

Rutaceae is another family with several members that provide edible fruits. Some of these include pomelo (*Citrus maxima*), grapefruit (*Citrus paradisi*), orange (bitter orange—*Citrus aurantium*; sweet orange including Valencia orange and navel orange—*Citrus sinensis*), mandarin (*Citrus reticulata* or *Citrus nobilis*) including the tangerine, clementine, Satsuma mandarin, tangor, tangelo and the ugli fruit, lemon (*Citrus limon*), lime (*Citrus aurantifolia*), and citron (*Citrus medica*). Pomelo and citron are very large weighing over 10 lb. The citrus fruits can be stored in a cool dry place. For longer shelf life, they can be stored between 35 and 50°F (between 45 and 50°F for grapefruit) in well-ventilated bags.

2.4.3 Ericaceae

Blueberry (lowbush blueberry—*Vaccinium angustifolium*; highbush blueberry—*Vaccinium corymbosum*) and bilberry (*Vaccinium myrtillus*) are important members. These berries are rich in anthocyanins, have strong antioxidant property, and are considered as functional foods. The berries are also moderately acidic and not very sweet, and hence ideal for diabetic population. The berries are very fragile and do not store very well for long periods. The ripe berries are harvested, and ideally, they can be frozen to preserve the nutrients as soon as they are picked. Cranberry fruits (*Vaccinium macrocarpon* and *Vaccinium oxycoccos*), on the other hand, can be stored in perforated plastic bags for a considerable period in the cold or stored frozen. The fruits are processed into juice, sauce, fruit flavored pieces, etc. Just as blueberry and bilberry, cranberries are very rich in anthocyanins and proanthocyanidins. They are strong antioxidants as well as help prevent urinary tract infections.

2.4.4 Vitaceae

Grapes are one of the major fruits consumed fresh and processed into wine, jams and jellies, powder, raisins, etc. Grapes are one of the oldest cultivated plants known, dating back to over 7,000 years. There are three major species: the European grapes (*Vitis vinifera*) or their grafts that are cultivated around the world for producing wine grapes, the North American grapes (*Vitis labrusca* and *Vitis rotundifolia*), and the hybrid grapes. Grapes are primarily used for winemaking, fresh consumption, or producing raisins apart from other processing uses such as juice and jam production. Varieties such as the Concord (purple black), the Niagara (green), and the Catawba (red) are *labrusca* varieties. Several locally produced varieties exist in different parts of the world. Some of the European varieties include the Cardinal, the Muscat, and the Lival, which are blue or black grapes. Common North American varieties include the Concord, the Flame, the Ruby (red seedless), and Thompson (green seedless). The skin becomes easily separated from the pulp in the North American grapes. The grapes do not store very well and may undergo anaerobic fermentation if stored too long. They are processed immediately. Table grapes can be stored in the cold in perforated plastic bags for 1–2 months.

2.4.5 Other fruit families

Some of the other common dicot fruits include cherimoya (*Annona cherimola*, *Annona squamosa*, Annonaceae), durian (*Durio zibethinus*, Bombaceae), jackfruit (*Artocarpus heterophyllus*, Moraceae), rambutan (*Nephelium lappaceum*, Sapindaceae), litchi (*Litchi chinensis*, Sapindaceae), longan (*Dimocarpus longan,* Sapindaceae), persimmon (*Diospyros virginiana*, *D. kaki*, Ebenaceae), papaya (*Carica papaya*, Caricaceae), kiwi fruit (*Actinidia chinensis*, Actinidiaceae), pomegranate (*Punica granatum*, Punicaceae), guava (*Psidium guayava*, Myrtaceae), mangosteen (*Garcinia mangostana*, Guttiferae), mango (*Mangifera indica*, Anacardiaceae), sapodilla (sapota) (*Manilkara sapota*, Sapotaceae), and currants (*Ribes rubrum*, *Ribes sativum*, *Ribes vulgare*, etc.; red currant; *Ribes nigrum*—black currant; Saxifragaceae). These fruits have characteristic qualities.

Annona fruits are highly flavorful and are highly perishable. Ripe fruits become damaged within days and produce off-flavors. The fruits are sometimes picked before ripening for transportation. Durian fruits are large (~10–15 lb), thorny, resembling jackfruits and are cultivated primarily in Southeast Asia and are well known for the foul smell of the fruits, composed by a variety of compounds including organosulfur compounds. Jackfruits are popular in South India and Sri Lanka. They are thorny on the outside and when ripened are highly flavorful. The fruits on some trees can weigh over 100 lb. The fruits contain latex, which can be removed by edible oils such as coconut oil. The fruit is an aggregate fruit with several fruitlets containing seeds that are rich in protein and can be consumed after cooking. Ripe fruits do not store well for more than 3–4 days uncut. Rambutan, again from Southeast Asia, is a 2–3 inches long hairy fruit and is highly perishable. The pulpy interior is edible. Litchi, originally cultivated in southern China, is a popular fruit. Removing the leathery skin in ripe fruits exposes a fleshy interior that is edible. Litchis have a reasonably long shelf life, but are best when consumed fresh. Papaya, originally native to South America, is cultivated worldwide. The flesh of ripe fruits is initially hard, but becomes soft as ripening progresses. The skin and flesh contain a latex that contains proteases, and young fruits are sometimes used as meat tenderizers. Flesh from mature fruits can also be cooked and consumed. Papayas also contain heterocyclic compounds similar to piperazine and are sometimes consumed to protect from intestinal worms. Ripe fruits of papaya have a very short shelf life. At present, kiwi fruit is widely cultivated around the world. The fruit is a berry, 3–4 inches long, and is covered by a brownish skin. The green flesh along with the seeds in ripened fruits can be eaten and tastes slightly acidic and tart. Unripe fruits can be stored for 2–3 weeks. The kiwi fruits also contain proteases and can be used as a meat tenderizer. Pomegranate, with a history of over 4,000 years, is cultivated widely and is well known for its medicinal properties. The fruits are covered with a leathery skin containing multiple chambers filled with juicy pulpy red crimson–colored seeds that are sweet with slight sour tart taste. The skin contains proanthocyanidins and polyphenols having very strong antioxidant activity. Alhough not consumed, the skin is commonly used in traditional medicine in India. Pomegranates have good postharvest shelf life (2–3 weeks at RT) and are easy to handle. Guava is becoming more popular with several new varieties. The ripe fruits have a pleasant flavor and sweet taste. The seeds are hard, and new seedless varieties are being cultivated. The ripe fruits have a very short shelf life. Some varieties have a red-colored interior like a melon and are rich in carotenoids. Mango has become an increasingly accepted fruit in the Western world. Several varieties

with excellent quality characteristics are available. Mango fruits have an extremely pleasant flavor and ripe fruits are very sweet. The shelf life of ripe fruits at RT may not exceed a week. The fruits of sapota are similar to kiwi fruits in appearance, with a leathery brown skin. When ripened, the fruits have a pleasant flavor and are extremely sweet. The fruits have a 1–2 weeks' shelf life at RT. The currants are nutritionally rich (vitamin C, minerals, anthocyanins) fruits gaining in popularity. The fruits are sour and are processed for juice or jams. The ripe fruits will stay on the plant for 2–3 weeks. Once harvested, they decay very fast.

Some of the other major fruit crops include plantains (*Musa paradisiaca*, Musaceae), pineapple (*Ananas comosus*, Bromeliaceae), and dates (*Phoenix dactylifera*, Palmaceae). The starchy interior of plantains is consumed in several African countries as a staple food. The mature fruit ripens after harvest or on the plant and does not store well. The fruit overripens and decays within a week. The ripe fruit is soft and rich in sugar and pectin. Pineapples have become very popular as an edible fruit, and a large amount of harvested fruits are processed. The fruits are harvested when mature, and during ripening, the stored organic acids are converted to sugars. Again, pineapple may store for 2–3 weeks when kept in a cool room. Date fruits, on the other hand, can be stored for a considerable length of time and are traditionally transported to several countries from the Middle East.

2.5 Vegetables

2.5.1 *Allium* vegetables

Allium vegetables belong to the family Liliaceae. These include chive (*Allium schoenoprasum* and *Allium tuberosum*), leeks (*A. porum*), garlic (*Allium sativum*), onion (*Allium cepa*), and shallot (*Allium ascalonicum*). Native to Central Asia, onions are perhaps the largest consumed of these vegetables and have been in cultivation for over 5,000 years. Onions are biennials, cultivated as annuals, and harvested at various stages of maturity to provide green leafy vegetables or when matured to provide the bulbs. During growth and maturation, food is stored in the leaf to form the fleshy, juicy edible bulb. The pigmentation in the skin in dry onion bulbs characterizes them into red, purple, yellow, brown, and white varieties with differing pungency, taste, and flavor. White and red onions are mild and sweet. Onions are harvested when the leaves wilt and outer leaf layers of the bulb become dry.

After harvest, the dry onions are dormant for a period, and depending on the storage period required, onions may be irradiated to prevent sprouting. The shelf life is longer in pungent varieties as the sulfur components may help deter the attack by pathogens. The yellow onions have a shelf life of 2–3 months in a cool dry place, whereas the red onions have a shelf life of only 2–4 weeks. Onions are not refrigerated or stored along with potatoes.

Garlic is another member of the *Allium* family with well-denoted properties of taste, flavor, and pungency. It is widely used for its medicinal properties. The bulb is a cluster of "cloves" that may vary in number. Although white garlic is common, there are varieties with pink and purple color on the skin. Garlic is best stored around 0°C at a relative humidity not greater than 60%, and can give a shelf life of 6 months or more. Chives and leeks can be stored for 2 weeks in the refrigerator.

A characteristic feature of the *Allium* family is its pungency. This property originates from the presence of sulfur-containing compounds. These precursor components such as

S-alk(en)yl cysteine sulfoxide and sulfonic acid are stored in a compartmentalized form stored in the vacuole. When the cells are ruptured, the precursor molecules react with a cytosolic enzyme allinase, releasing a number of volatile components that include thiosulfinates, thiosulfonates, disulfides, and sulfonic acids. Allicin, alliin, diallylsulfide, and ajoene are volatile components present in onion and garlic products. Lachrymatory factors (thiopropanal *S*-oxide, C_2H_5—CH═SO) are generated by the action of allinase on the precursor molecules such as allicin and alliin (Block et al., 1992). Despite their pungent nature, *Allium* vegetables contain several nutraceuticals, and consumption of these vegetables, in general, is regarded as a healthy choice.

2.5.2 Crucifer vegetables

Just as the *Allium* family, Cruciferae is rich in vegetables with several nutraceuticals showing wide-ranging health benefits. Some of the commonly used crucifer vegetables include turnip (*Brassica rapa*), radish (*Raphanus sativus*), rutabaga (*Brassica napus* var. napobrassica), cabbage (*Brassica oleracea*), kohlrabi (*B. oleracea*), cauliflower (*B. oleracea* var. botrytis), and broccoli (*B. oleracea* var. italica). Most of the crucifer vegetables appear to have originated in the Mediterranean and was consumed over 4,000 years ago by the Egyptians and Babylonians. There are several types of radishes that include the red, black, and white radish. The radish stores the essential oil in the surface cell layers of the tuber, which provides the pungency to the vegetable. The radishes are best stored unwashed in perforated plastic bags around 4°C after removal of the leaves and usually stores for a week to 10 days. Turnips have a better storage life. Rutabaga (*rotabaggar* in Swedish) originated as a cross between savoy cabbage and turnip in Scandinavia and was used as a staple food source during food scarcity. It is more pungent than turnip, and can be stored in a perforated plastic bag at 4°C or for months if waxed. Cabbages, cauliflower, and broccoli are potentially the most widely used crucifer vegetables today, leaves being the nutritional source in cabbage and florets being the nutritional source in cauliflower and broccoli. Cauliflower can be stored unwashed in a perforated plastic bag at 4°C for a week to 10 days. Broccoli is highly perishable, and the quality deteriorates rapidly even at low temperatures within days. Controlled atmosphere storage can extend the shelf life of broccoli over 2 months.

2.5.3 Umbelliferae vegetables

Umbelliferae (Umbellaceae) is another vegetable-rich family. In addition to the tuberous vegetables, the seeds from the plants are valuable food sources with health regulatory properties. The major vegetables of this family include parsnip (*Pastinaca sativa*), carrot (*Daucus carota* var. sativa), celeriac (*Apium graveolens* var. rapaceum), celery (*Apium graveolens* var. dulce), and fennel (*Foeniculum vulgare*). Carrot and parsnip roots can be stored for a considerably long period in cold. Carrots can be stored for over 6 months at near-zero temperatures. Storing along with fruits and vegetables that evolve ethylene gas causes the development of bitter taste in carrots. The leaf bases of celery and fennel are used as vegetables and have a shelf life of 1–2 weeks in the refrigerator. Celeriac, often called celery roots, can be stored refrigerated for several weeks.

2.5.4 Cucurbits

Cucurbits are another major group of vegetables belonging to Cucurbitaceae that are cultivated widely in the tropics and subtropics. The fruits are varied in nature, but all originate from an inferior ovary. Some cucurbits such as the cucumber (*Cucumis sativus*) and melons (*Cucumis melo*) are consumed raw. The melons are fruits rather than vegetables. There are several varieties of gourds and squashes. Some of these include wax gourd (*Benincasa hispida*), bitter gourd (melon) (*Momordica charantia*), squash (zucchini—*Cucumis pepo*), winter squash (butternut squash—*Cucumis moschata*; turban squash and buttercup squash—*C. maxima* var. turbaniformis; banana squash—*C. maxima*), pumpkin (*C. pepo*), and sphaghetti squash (*C. pepo*). Chayote (*Sechium edule*) is common in Mexico and Central America. The ripened pumpkins, squashes, and some cucumbers can be stored for months in a cool dry place. The cucurbits are chilling sensitive and low-temperature storage is not required. The bitter gourd, as the name suggests, is very bitter and does not store well. Water loss is a problem even at low temperature, and the vegetable is best used within a week of harvest. In general, young immature fruits are harvested for consumption. Several cucurbits are considered to have medicinal properties such as the bitter gourd (against type 2 diabetes).

2.5.5 Solanaceous vegetables

Several members of Solanaceae are important vegetables, though the family is associated with poisonous alkaloids. The major solanaceous vegetable crops include tomato (*Solanum lycopersicum*, formerly *Lycopersicon esculentum*), potato (*Solanum tuberosum*), eggplant (*Solanum melongena*), and sweet and hot peppers (*Capsicum annuum*). The eggplants are consumed when young and never used after ripening, whereas tomatoes are used after ripening. The peppers can be used before or after ripening depending on the type. Potato is the exception in this family, and the edible part is the stem tuber. Solanaceous fruits do not store very well. In general, a postharvest life of 7–10 days can be anticipated during refrigerated storage. Potatoes, on the other hand, can be stored for months covered with dry soil in a cool place or under a controlled atmosphere at a low temperature.

2.5.6 Compositae vegetables

The family of Compositae (Asteraceae) includes several leafy vegetables. Some of these include wild chicory or endive (*Cichorium intybus*), curly chicory (*Cichorium endivia*), lettuce (*Lactuca sativa*), and artichoke (*Cyanara scolymus*). Jerusalem artichoke (*Helianthus tuberosus*) is an exception and produces an edible tuber, though not widely used at present. Lettuce is one of the most popular vegetables. There are several varieties of lettuce including the head lettuce, butterhead lettuce, leaf lettuce, and romaine lettuce. The shelf life of leafy vegetables is low. These can best be stored in perforated plastic bags in the refrigerator for a week. Leaf and romaine lettuce should be washed to get rid of the soil and excess moisture removed before storing. Some varieties such as Boston lettuce and iceberg lettuce are very fragile and are washed just before consumption. They can be stored in an airtight compartment in the refrigerator and should not be kept together with ethylene-producing fruits since the ethylene tends to promote browning in lettuce.

2.5.7 Legume vegetables

Leguminosae (Fabaceae) is an important family having a number of vegetables. The seed-bearing pods of most leguminous vegetables are edible as fresh commodities, but can also be allowed to mature when these can be dried to provide a variety of pulses that are an integral part of the diet in many continents. The leguminous vegetables and pulses are excellent sources of carbohydrates and protein. Some of the fresh edible legume vegetables include beans (*Phaseolus* sp., *Dolichos lablab*, *Vigna* sp.), broad bean (*Vicia faba*), and peas (*Pisum sativum*). Dried seeds from these vegetables are also used. The pods of other leguminous members such as mung bean (*Phaseolus aureus* or *Vigna radiata*), black gram (*Phaseolus mungo* or *Vigna mungo*), runner beans (*Phaseolus coccineus*), lentils (*Lens culinaris* or *Lens esculenta*), chickpea (*Cicer arietinum*), and soybean (*Glycine max*) are not consumed fresh, but the seeds are allowed to mature and dry before harvesting. Immature pods of peanuts (*Arachis hypogea*) are sometimes steamed and the seeds consumed before they are matured, though the matured seeds are the major products. The fresh seedpods do not store well and they become dehydrated very fast. The fresh pods have better quality when consumed within 2–3 days of harvest. Some varieties can be stored in a refrigerator for a week if they do not develop chilling-injury symptoms.

2.5.8 Tuber vegetables

Tuber vegetables store starch and are staple foods in many parts of the world. These belong to several different families. Some of these vegetables include beet (*Beta vulgaris*, Chenopodiaceae), malanga (*Xanthosoma sagittifolium*, Araceae), taro (*Colocasia esculenta*, Araceae), cassava or tapioca (*Manihot esculenta*, *Manihot dulcis*, Euphorbiaceae), yam (*Dioscorea* sp.), and sweet potato (*Ipomea batatas*, Convolvulaceae). Most of these tubers can be stored in a cool dry place for months without special treatments. Cassava roots are waxed to extend the shelf life for over a month.

2.6 Flowers

In contrast to fruits and vegetables, the number of cultivated flowers is very small. The major flower crops include roses (*Rosa* sp., Rosaceae), carnations (*Dianthus caryophyllus*, Caryophyllaceae), aster, daisies and *Chrysanthemum* (Asteraceae), snapdragons (Leguminosae), *Gladiolus*, tulips, lilies, and *Alstroemeria* (Liliaceae). In recent years, the cultivation of potted ornamentals has gained importance. Some of the potted ornamentals include miniroses, *Gerbera*, kalanchoe, various orchids, *Chrysanthemums*, African violets, cyclamens, *Hydrangea, Poinsettia*, and various cacti. The potted ornamentals retain the quality much better than cut flowers and withstand the rigors of transportation better.

General reading

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Chapter 3

Biochemistry of Fruits

Gopinadhan Paliyath and Dennis P. Murr

3.1 Introduction

Several metabolic changes are initiated after the harvest of fruits and vegetables. In the case of vegetables, harvesting induces stress responses through reduced availability of water and nutrients, wounding and exposure to shelf life, enhancing storage methods such as cooling. In most cases, these changes help the produce to enhance the shelf life. In the case of fruits, an increase in the biosynthesis of the gaseous hormone ethylene serves as the physiological signal for the initiation of the ripening process. In general, all plant tissues produce a low, basal, level of ethylene. During the ripening process, some fruits evolve large amounts of ethylene, sometimes referred to as an autocatalytic increase in ethylene production, which occurs in conjunction with an increase in respiration referred to as the respiratory climacteric. Fruits are generally classified into climacteric or nonclimacteric types on the basis of the pattern of ethylene production and responsiveness to externally added ethylene. The climacteric fruits characteristically show a marked enhancement in ethylene production and respiration, as noticeable by the evolution of carbon dioxide. By contrast, the nonclimacteric fruits emit a considerably reduced level of ethylene. (For a list of fruits showing climacteric or nonclimacteric pattern of ripening, see Kays (1997), General Reading.) In climacteric fruits such as apple, pear, banana, tomato, and avocado, ethylene evolution can reach 30–500 ppm/(kg h) (parts per million, microliter/L), whereas in nonclimacteric fruits such as orange, lemon, strawberry, and pineapple, ethylene levels usually range from 0.1 to 0.5 ppm/(kg h) during ripening. Climacteric fruits respond to external ethylene treatment by an early induction of the respiratory climacteric and accelerated ripening in a concentration-dependent manner. Nonclimacteric fruits, on the other hand, show increased respiration in response to increased levels of ethylene concentration without showing acceleration in the time required for ripening. Vegetables produce very low amounts of ethylene most of them with less than 0.1 μL/(kg h), with slightly higher levels as in cassava (1.7 μL/kg h), breadfruit (1.2 μL/kg h), and cucumber (0.6 μL/kg h) when measured at 20–25°C.

In all plants, ethylene biosynthesis occurs through a common pathway that uses the sulfur amino acid methionine as the precursor (Yang, 1981; Fluhr and Mattoo, 1996) (Fig. 3.1). The first reaction of the pathway involves the conversion of methionine to *S*-adenosyl methionine (SAM) mediated by the enzyme methionine adenosyl transferase. SAM is in turn converted into 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase. The sulfur moiety of methylthioribose generated during this reaction

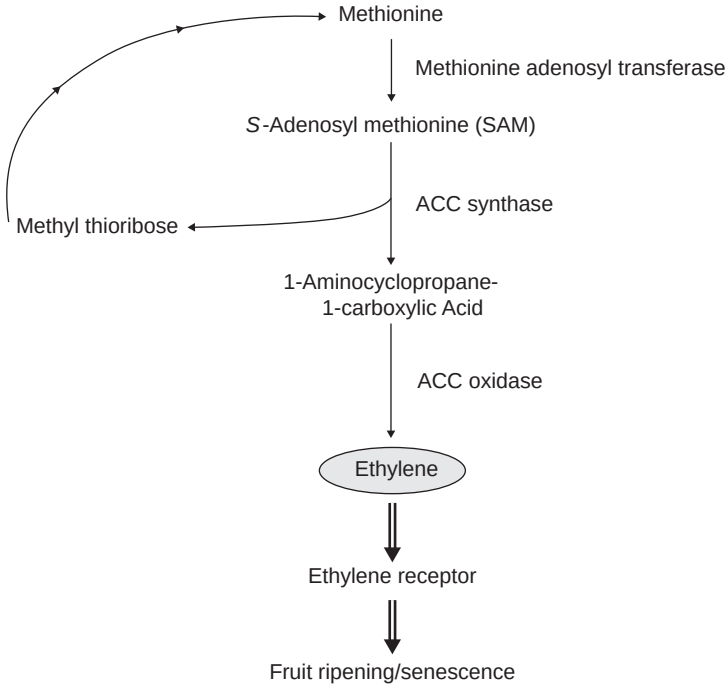


Fig. 3.1 Ethylene biosynthetic pathway in plants. ACC synthase, aminocyclopropane carboxylic acid synthase; ACC oxidase, aminocyclopropane carboxylic acid oxidase.

is reused during the biosynthesis of methionine. The immediate precursor of ethylene is ACC, which undergoes oxidation by ACC oxidase to generate ethylene. Biochemical steps involving ACC synthase and ACC oxidase are the key regulatory points in the biosynthesis of ethylene. ACC synthase that is localized in the cytoplasm is a soluble enzyme with a relative molecular mass of 50 kDa (kilodalton). ACC synthase has several isoforms that are differentially expressed in response to wounding, other stress factors, and at the initiation of ripening. In apple fruits, ACC oxidase was localized in the cytosol using immunoelectron microscopy (Chung et al., 2002). Using molecular biology tools, a cDNA (complementary DNA representing the coding sequences of a gene) for ACC oxidase was isolated from tomato, which encodes a protein with a relative molecular mass of 35 kDa (Hamilton et al., 1991). ACC-oxidase reaction occurs in the presence of Fe^{2+}, ascorbate, and oxygen.

Regulation of ethylene biosynthesis is a critical factor in the preservation of shelf life and quality in fruits. Controlled atmosphere storage under low oxygen reduces ethylene production. Ethylene scrubbing is also a common practice in storage facilities. Biotechnological approaches to reduce ethylene production by tissue through the regulation of the activities of ACC synthase and ACC oxidase have provided additional methods for the preservation of shelf life and quality in fruits. Some examples include the inhibition of ACC-synthase and ACC-oxidase gene expression through the introduction of their respective antisense cDNAs, which caused delayed ripening and resulted in better preservation of the quality of tomato (Hamilton et al., 1990; Oeller et al., 1991) and apple (Hrazdina

et al., 2000) fruits. ACC synthase is the rate-limiting enzyme of the ethylene biosynthetic pathway and requires pyridoxal-5-phosphate as a cofactor. ACC synthase is inhibited by pyridoxal phosphate inhibitors such as aminoethoxyvinylglycine and aminooxyacetic acid. Field application of aminoethoxyvinylglycine as a growth regulator (retain, valent chemicals) is a practical method of delaying the ripening in fruits such as apples, peaches, and pears. As well, controlled atmosphere storage at very low oxygen levels (1–3%) is a common practice in commercial operations for long-term storage of fruits such as apples to reduce the production of ethylene, since oxygen is required for the conversion of ACC to ethylene.

After the initiation of ripening or harvest, several biochemical changes occur in fruits and vegetables. As some of these changes such as the development of color, flavor, and sweet taste are desirable for fruits—any sort of quality changes are ideally not desired in vegetables. Thus, strategies for the preservation of shelf life and quality in fruits and vegetables could be entirely different. It is important to know the biochemical differences between fruits and vegetables and several biochemical pathways that operate in these tissues to develop ideal conditions of storage for the preservation of shelf life and quality.

3.2 Biochemical composition of fruits

Fruits contain a large percentage of water that can often exceed 95% by fresh weight. During ripening, activation of several metabolic pathways often leads to drastic changes in the biochemical composition of fruits. Fruits such as banana store starch during development and hydrolyze the starch to sugars during ripening that also results in fruit softening. Most fruits are capable of photosynthesis, store starch, and convert them to sugars during ripening. Fruits such as apple, tomato, and grape have a high percentage of organic acids, which decreases during ripening. Fruits contain large amounts of fibrous materials such as cellulose and pectin. The degradation of these polymers into smaller water-soluble units during ripening leads to fruit softening as exemplified by the breakdown of pectin in tomato and cellulose in avocado. Secondary plant products are major compositional ingredients in fruits. Anthocyanins are the major color components in grapes, blueberries, apples, and plums; carotenoids, specifically lycopene and carotene, are the major components that impart color in tomatoes. Aroma is derived from several types of compounds that include monoterpenes (as in lime, orange), ester volatiles (ethyl, methyl butyrate in apple, isoamyl acetate in banana), simple organic acids such as citric and malic acids (citrus fruits, apple), and small chain aldehydes such as hexenal and hexanal (cucumber). Fruits are also rich in vitamin C. Lipid content is quite low in fruits, the exceptions being avocado and olives, in which triacylglycerols (oils) form the major storage components. The amounts of proteins are usually low in most fruits.

3.2.1 Carbohydrates, storage, and structural components

As the name implies, carbohydrates are organic compounds containing carbon, hydrogen, and oxygen. Basically, all carbohydrates are derived by the photosynthetic reduction of CO_2, and the hexoses (glucose, fructose) and pentoses (ribose, ribulose) that are intermediates in the pathway are further converted to several sugar monomers. Polymerization of several

sugar derivatives leads to various storage (starch) and structural components (cellulose, pectin).

During photosynthesis, the glucose formed is converted to starch and stored as starch granules. Glucose and its isomer fructose, along with phosphorylated forms (glucose-6-phosphate, glucose-1,6-diphosphate, fructose-6-phosphate, and fructose-1,6-diphosphate), can be considered to be the major metabolic hexose pool components that provide carbon skeleton for the synthesis of carbohydrate polymers. Starch is the major storage carbohydrate in fruits. There are two molecular forms of starch—amylose and amylopectin—and both components are present in the starch grain. Starch is synthesized from glucose phosphate by the activities of a number of enzymes designated as ADP-glucose pyrophosphorylase, starch synthase and a starch-branching enzyme. ADP-glucose pyrophosphorylase catalyzes the reaction between glucose-1-phosphate and ATP that generates ADP-glucose and pyrophosphate. ADP-glucose is used by starch synthase to add glucose molecules to amylose or amylopectin chain, thus increasing their degree of polymerization. In contrast to cellulose that is made up of glucose units in β-1,4-glycosidic linkages, the starch molecule contains glucose linked by α-1,4-glycosidic linkages. The starch-branching enzyme introduces glucose molecules through α-1,6-linkages to a linear amylose molecule. These added glucose branch points serve as sites for further elongation by starch synthase, thus resulting in a branched starch molecule, also known as amylopectin.

Cell wall is a complex structure composed of cellulose and pectin, derived from hexoses such as glucose, galactose, rhamnose and mannose, and pentoses such as xylose and arabinose, as well as some of their derivatives such as glucuronic and galacturonic acids. A model proposed by Keegstra et al. (1993) describes the cell wall as a polymeric structure constituted by cellulose microfibrils and hemicellulose embedded in the apoplastic matrix in association with pectic components and proteins. In combination, these components provide the structural rigidity that is characteristic to the plant cell. Most of the pectin is localized in the middle lamella. Cellulose is biosynthesized by the action of β-1,4-glucan synthase enzyme complexes that are localized on the plasma membrane. The enzyme uses uridine diphosphate glucose (UDPG) as a substrate and, by adding UDPG units to small cellulose units, extends the length and polymerization of the cellulose chain. In addition to cellulose, there are polymers made of different hexoses and pentoses known as hemicelluloses, and based on their composition, they are categorized as xyloglucans, glucomannans, and galactoglucomannans. The cellulose chains assemble into microfibrils through hydrogen bonds to form crystalline structures. In a similar manner, pectin is biosynthesized from UDP-galacturonic acid (galacturonic acid is derived from galactose, a six-carbon sugar) as well as other sugars and derivatives and includes galacturonans and rhamnogalacturonans that form the acidic fraction of pectin. As the name implies, rhamnogalacturonans are synthesized primarily from galacturonic acid and rhamnose. The carboxylic acid groups complex with calcium, which provide the rigidity to the cell wall and the fruit. The neutral fraction of the pectin comprises polymers such as arabinans (polymers of arabinose), galactans (polymers of galactose), or arabinogalactans (containing both arabinose and galactose). All these polymeric components form a complex three-dimensional network stabilized by hydrogen bonds, ionic interactions involving calcium, phenolic components such as diferulic acid and hydroxyproline-rich glycoproteins (Fry, 1986). It is also important to visualize that these structures are not static and the components of cell wall are constantly being turned over in response to growth conditions.

3.2.2 Lipids and biomembranes

By structure, lipids can form both structural and storage components. The major forms of lipids include fatty acids, diacyl- and triacylglycerols, phospholipids, sterols, and waxes that provide an external barrier to the fruits. Fruits, in general, are not rich in lipids with the exception of avocado and olives that store large amounts of triacylglycerols or oil. As generally observed in plants, the major fatty acids in fruits include palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3) acids. Among these, oleic, linoleic, and linolenic acids possess an increasing degree of unsaturation. Olive oil is rich in triacylglycerols containing the monounsaturated oleic acid and is considered as a healthy ingredient for human consumption.

Compartmentalization of cellular ingredients and ions is an essential characteristic of all life forms. The compartmentalization is achieved by biomembranes, formed by the assembly of phospholipids and several neutral lipids that include diacylglycerols and sterols, the major constituents of the biomembranes. Virtually, all cellular structures include or are enclosed by biomembranes. The cytoplasm is surrounded by the plasma membrane, the biosynthetic, and the transport compartments such as the endoplasmic reticulum and golgi bodies form an integral network of membranes within the cell. Photosynthetic activity, which converts light energy into chemical energy, and respiration, which further converts chemical energy into more usable forms, occur on the thylakoid membrane matrix in the chloroplast and the cristae of mitochondria, respectively. All these membranes have their characteristic composition and enzyme complexes to perform their designated function.

The major phospholipids that constitute the biomembranes include phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylinositol. Their relative proportion may vary from tissue to tissue. In addition, metabolic intermediates of phospholipids such as phosphatidic acid, diacylglycerols, and free fatty acids are also present in the membrane in lower amounts. Phospholipids are integral functional components of hormonal and environmental signal transduction processes in the cell. Phosphorylated forms of phosphatidylinositol such as phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate are formed during signal transduction events, though their amounts can be very low. The membrane also contains sterols such as sitosterol, campesterol, and stigmasterol, as well as their glucosides, and they are extremely important for the regulation of membrane fluidity and function.

Biomembranes are bilamellar layers of phospholipids. The amphipathic nature of phospholipids having hydrophilic head groups (choline, ethanolamine, etc.) and hydrophobic fatty acyl chains thermodynamically favor their assembly into bilamellar or micellar structures when exposed to an aqueous environment. In a biomembrane, the hydrophilic headgroups are exposed to the external aqueous environment. The phospholipid composition between various fruits may differ, and within the same fruit, the inner and outer lamella of the membrane may have a different phospholipid composition. Such differences may cause changes in polarity between the outer and inner lamellae of the membrane and lead to the generation of a voltage across the membrane. These differences usually become operational during signal transduction events.

An essential characteristic of the membrane is its fluidity. The fluid-mosaic model of the membrane (Singer and Nicholson, 1972) depicts the membrane as a planar matrix composed of phospholipids and proteins. The proteins are embedded in the membrane

bilayer (integral proteins) or are bound to the periphery (peripheral proteins). The nature of this interaction stems from the structure of the proteins. If the proteins have a much larger proportion of hydrophobic amino acids, they would tend to become embedded in the membrane bilayer. If the protein contains more hydrophilic amino acids, it may tend to prefer a more aqueous environment and thus remain as a peripheral protein. In addition, proteins may be covalently attached to phospholipids such as phosphatidylinositol. Proteins that remain in the cytosol may also become attached to the membrane in response to an increase in cytosolic calcium levels. The membrane is a highly dynamic entity. The semifluid nature of the membrane allows for the movement of phospholipids in the plane of the membrane and between the bilayers of the membrane. The proteins are also mobile within the plane of the membrane. However, this process is not always random and is regulated by the functional assembly of proteins into metabolons (photosynthetic units in thylakoid membrane, respiratory complexes in the mitochondria, cellulose synthase on plasma membrane, etc.), their interactions with the underlying cytoskeletal system (network of proteins such as actin and tubulin), and the fluidity of the membrane.

The maintenance of homeostasis (life processes) requires the maintenance of the integrity and function of discrete membrane compartments. This is essential for the compartmentalization of ions and metabolites, which may otherwise destroy the cell. For instance, calcium ions are highly compartmentalized within the cell. The concentration of calcium is maintained at the millimolar levels within the cell wall compartment (apoplast), endoplasmic reticulum, and the tonoplast (vacuole). This is achieved by energy-dependent extrusion of calcium from the cytoplasm into these compartments by ATPases. As a result, the cytosolic calcium levels are maintained at low micromolar ($<1\ \mu$M) levels. Maintenance of this concentration gradient across the membrane is a key requirement for the signal transduction events, as regulated entry of calcium into the cytosol can be achieved simply by opening calcium channels. Calcium can then activate several cellular biochemical reactions that mediate the response to the signal. Calcium is pumped back into the storage compartments when the signal diminishes in intensity. In a similar manner, cytosolic pH is highly regulated by the activity of proton ATPases. The pH of the apoplast and the vacuole is maintained near 4, whereas the pH of the cytosol is maintained in the range of 6–6.5. The pH gradient across the membrane is a key feature that regulates the absorption or extrusion of other ions and metabolites such as sugars. The cell could undergo senescence if this compartmentalization is lost.

There are several factors that affect the fluidity of the membrane. The major factor that affects the fluidity is the type and proportion of acyl chain fatty acids of the phospholipids. At a given temperature, a higher proportion of unsaturated fatty acyl chains (oleic, linoleic, linolenic) in the phospholipids can increase the fluidity of the membrane. An increase in saturated fatty acids such as palmitic and stearic acids can decrease the fluidity. Other membrane components such as sterols and degradation products of fatty acids such as fatty aldehydes and alkanes can also decrease the fluidity. Based on the physiological status of the tissue, the membranes can exist in either a liquid crystalline state (where the phospholipids and their acyl chains are mobile) or a gel state where they are packed as rigid-ordered structures and their movements are much restricted. The membrane usually has coexisting domains of liquid crystalline and gel-phase lipids depending on growth conditions, temperature, ion concentration near the membrane surface. The tissue has the ability to adjust the fluidity of the membrane by altering the acyl lipid composition of the phospholipids. For instance, an increase in the gel-phase lipid domains resulting from

exposure to cold temperature could be counteracted by increasing the proportion of fatty acyl chains having a higher degree of unsaturation and therefore a lower melting point. Thus, the membrane will tend to remain fluid even at a lower temperature. An increase in gel-phase lipid domains can result in the loss of compartmentalization. The differences in the mobility properties of phospholipid acyl chains can cause packing imperfections at the interface between gel and liquid crystalline phases, and these regions can become leaky to calcium ions and protons that are highly compartmentalized. The membrane proteins are also excluded from the gel phase into the liquid crystalline phase. Thus, during examinations of membrane structure by freeze fracture electron microscopy, the gel-phase domains can appear as regions devoid of proteins (Paliyath and Thompson, 1990).

3.2.3 Proteins

Fruits, in general, are not very rich sources of proteins. During the early growth phase of fruits, the chloroplasts and mitochondria are the major organelles that contain structural proteins. The structural proteins include the light-harvesting complexes in chloroplast or the respiratory enzyme/protein complexes in mitochondria. Ribulose-bis-phosphate carboxylase/oxygenase (Rubisco) is the most abundant enzyme in photosynthetic tissues. Fruits do not store proteins as an energy source. The green fruits such as bell peppers and tomato have a higher level of chloroplast proteins.

3.2.4 Organic acids

Organic acids are major components of fruits. The acidity of fruits arises from the organic acids that are stored in the vacuole, and their composition can vary depending on the type of fruit. In general, young fruits contain more acids that may decline during maturation and ripening due to their conversion to sugars (gluconeogenesis). Some fruit families are characterized by the presence of certain organic acids. For example, fruits of Oxalidaceae members (ex. Starfruit, *Averrhoa carambola*) contain oxalic acid, and fruits of the citrus family, Rutaceae, are rich in citric acid. Apples contain malic acid and grapes are characterized by the presence of tartaric acid. In general, citric and malic acids are the major organic acids of fruits. Grapes contain tartaric acid as the major organic acid. During ripening, these acids can enter the citric acid cycle and undergo further metabolic conversions.

L-(+)tartaric acid is the optically active form of tartaric acid in grape berries. A peak in acid content is observed before the initiation of ripening, and the acid content declines on a fresh weight basis during ripening. Tartaric acid can be biosynthesized from carbohydrates and other organic acids. Radiolabeled glucose, glycolate, and ascorbate were all converted to tartarate in grape berries. Malate can be derived from the citric acid cycle or through carbon dioxide fixation of pyruvate by the malic enzyme (nicotinamide adenine dinucleotide phosphate (NADPH)-dependent malate dehydrogenase). Malic acid, as the name implies, is also the major organic acid in apples.

3.3 Fruit ripening and softening

Fruit ripening is the physiological repercussion of a very complex and interrelated biochemical changes that occur in the fruits. Ripening is the ultimate stage of the development

of the fruit, which entails the development of ideal organoleptic characters such as taste, color, and aroma that are important features of attraction for the vectors (animals, birds, etc.) responsible for the dispersal of the fruit, and thus the seeds, in the ecosystem. Human beings have developed an agronomic system of cultivation, harvest, and storage of fruits with ideal food qualities. In most cases, the ripening process is very fast, and the fruits undergo senescence resulting in the loss of desirable qualities. An understanding of the biochemistry and molecular biology of the fruit ripening process has resulted in developing biotechnological strategies for the preservation of postharvest shelf life and quality of fruits.

In response to the initiation of ripening, several biochemical changes are induced in the fruit, which ultimately results in the development of ideal texture, taste, color, and flavor. Several biochemical pathways are involved in these processes as described next.

3.3.1 Carbohydrate metabolism

3.3.1.1 Cell wall degradation

Cell wall degradation is the major factor that causes softening of several fruits. This involves the degradation of cellulose components, pectin components, or both. Cellulose is degraded by the enzyme cellulase or β-1,4-glucanase. Pectin degradation involves the enzymes pectin methylesterase, polygalacuronase (pectinase), and β-galactosidase. The degradation of cell wall can be reduced by the application of calcium as a spray or drench in apple fruits. Calcium binds and cross-links the free carboxylic groups of polygalacturonic acid components in pectin. Calcium treatment therefore also enhances the firmness of the fruits.

The activities of both cellulase and pectinase have been observed to increase during ripening of avocado fruits and result in their softening. Cellulase is an enzyme with a relative molecular mass of 54.2 kDa and formed by extensive posttranslational processing of a native 54-kDa protein involving proteolytic cleavage of the signal peptide and glycosylation (Bennet and Christofferson, 1986). Further studies have shown three isoforms of cellulose ranging in molecular masses between 50 and 55 kDa. These forms are associated with the endoplasmic reticulum, the plasma membrane, and the cell wall (Dallman et al., 1989). The cellulase isoforms are initially synthesized at the style end of the fruit at the initiation of ripening, and the biosynthesis moves toward the stalk end of the fruit with the advancement of ripening. Degradation of hemicelluloses (xyloglucans, glucomannans, and galactoglucomannans) is also considered as an important feature that leads to fruit softening. Degradation of these polymers could be achieved by cellulases and galactosidases.

Loss of pectic polymers through the activity of polygalacturonases (PG) is a major factor involved in the softening of fruits such as tomato. There are three major isoforms of polygalacturonases responsible for pectin degradation in tomato, designated as PG1, PG2a, and PG2b (Fischer and Bennet, 1991). PG1 has a relative molecular mass of 100 kDa, and is the predominant form at the initiation of ripening. With the advancement of ripening, PG2a and PG2b isoforms increase, becoming the predominant isoforms in ripe fruit. The different molecular masses of the isozymes result from the posttranslational processing and glycosylation of the polypeptides. PG2a (43 kDa) and PG2b (45 kDa) appear to be the same polypeptide with different degrees of glycosylation. PG1 is a complex of three polypeptides: PG2a, PG2b, and a 38-kDa subunit known as the β-subunit. The 38-kDa subunit is believed to exist in the cell wall space where it combines with PG2a and PG2b, forming the PG1

isoform of PG. The increase in activity of PG1 is related to the rate of pectin solubilization and tomato fruit softening during the ripening process.

Research into the understanding of the regulation of biosynthesis and activity of PG using molecular biology tools has resulted in the development of strategies for enhancing the shelf life and quality of tomatoes. PG mRNA is one of the first ripening-related mRNAs isolated from tomato fruits. All the different isoforms of PGs are encoded by a single gene. The PG cDNA which has an open reading frame of 1,371 bases encodes a polypeptide having 457 amino acids, that includes a 24-amino acid signal sequence (for targeting to the cell wall space) and a 47-amino acid prosequence at the N-terminal end, which are proteolytically removed during the formation of the active PG isoforms. A 13-amino acid long C-terminal peptide is also removed resulting in a 373-amino acid long polypeptide, which undergoes different degrees of glycosylation resulting in the PG2a and PG2b isozymes. Complex formation between PG2a, PG2b, and the 38-kDa subunit in the apoplast results in the PG1 isozyme (Grierson et al., 1986; Bird et al., 1988). In response to ethylene treatment of mature green tomato fruits, which stimulates ripening, the levels of PG mRNA and PG are found to increase. These changes can be inhibited by treating tomatoes with silver ions, which interfere with the binding of ethylene to its receptor and initiation of ethylene action (Davies et al., 1988). Thus, there is a link between ethylene, PG synthesis, and fruit softening.

Genetic engineering of tomato with the objective of regulating PG activity has yielded complex results. In the *rin* mutant of tomato, which lacks PG and does not soften, introduction of a PG gene resulted in the synthesis of an active enzyme; however, this did not cause fruit softening (Giovannoni et al., 1989). As a corollary to this, introduction of the PG gene in the antisense orientation resulted in near total inhibition of PG activity (Smith et al., 1988). In both these cases, there was very little effect on fruit softening, suggesting that factors other than pectin depolymerization may play an integral role in fruit softening. Further studies using tomato cultivar such as UC82B (Kramer et al., 1992) showed that antisense inhibition of ethylene biosynthesis or PG did indeed result in lowered PG activity, improved integrity of cell wall, and increased fruit firmness during fruit ripening. As well, increased activity of pectin methylesterase, which removes the methyl groups from esterified galacturonic acid moieties, may contribute to the fruit softening process.

The activities of pectin-degrading enzymes have been related to the incidence of physiological disorders such as “mealiness” or “wooliness” in mature unripened peaches that are stored at a low temperature. The fruits with such a disorder show a lack of juice and a dry texture. Deesterification of pectin by the activity of pectin methyl esterase is thought to be responsible for the development of this disorder. Pectin methyl esterase isozymes with relative molecular masses in the range of 32 kDa have been observed in peaches, and their activity increases after 2 weeks of low-temperature storage. Polygalacuronase activity increases as the fruit ripens. The ripening fruits that possess both polygalacturonase and pectin methyl esterase do not develop mealy symptoms when stored at low temperature, implicating the potential role of pectin degradation in the development of mealiness in peaches.

There are two forms of polygalacturonases in peaches: the exo- and endopolygalacturonases. The endopolygalacturonases (endo-PG) are the predominant forms in the freestone type of peaches, whereas the exopolygalacturonases (exo-PG) are observed in the mesocarp of both freestone and clingstone varieties of peaches. As the name implies, exopolygalacturonases remove galacturonic acid moieties of pectin from the terminal reducing end of the

chain, whereas the endopolygalacturonases can cleave the pectin chain at random within the chain. The activities of these enzymes increase during the ripening and softening of the fruit. Two exo-PG isozymes have been identified in peach, having a relative molecular mass of near 66 kDa. The exo-acting enzymes are activated by calcium. Peach endo-PG is observed to be similar to the tomato endo-PG. The peach endo-PG is inhibited by calcium. The freestone peaches possess enhanced activities of both exo-PG and endo-PG leading to a high degree of fruit softening. However, the clingstone varieties with low levels of endo-PG activity do not soften as the freestone varieties. In general, fruits such as peaches, tomatoes, strawberries, and pears, which soften extensively, possess high levels of endo-PG activity. Apple fruits, which remain firm, lack endo-PG activity.

3.3.1.2 Starch degradation

Starch is the major storage form of carbohydrates. During ripening, starch is catabolized into glucose and fructose, which enters the metabolic pool where they are used as respiratory substrates or further converted to other metabolites (Fig. 3.2). In fruits such as banana, the breakdown of starch into simple sugars is associated with fruit softening. There are several enzymes involved in the catabolism of starch. α-Amylase hydrolyzes amylose molecules by cleaving the α-1,4-linkages between sugars, providing smaller chains of amylose termed as dextrins. β-Amylase is another enzyme that acts on the glucan chain, releasing maltose, which is a diglucoside. The dextrins as well as maltose can be further catabolized to simple glucose units by the action of glucosidases. Starch phosphorylase is another enzyme, which mediates the phosphorolytic cleavage of terminal glucose units at the nonreducing end of the starch molecule using inorganic phosphate, thus releasing glucose-1-phosphate. The amylopectin molecule is also degraded in a similar manner to amylose, but also involves the action of debranching enzymes, which cleaves the α-1,6-linkages in amylopectin and releases linear units of the glucan chain.

In general, starch is confined to the plastid compartments of fruit cells, where it exists as granules made up of both amylose and amylopectin molecules. The enzymes that catabolize starch are also found in this compartment and their activities increase during ripening. The glucose-1-phosphate generated by starch degradation (Fig. 3.2) is mobilized into the cytoplasm where it can enter into various metabolic pools such as that of glycolysis (respiration), pentose phosphate pathway, or for turnover reactions that replenish lost or damaged cellular structures (cell wall components). It is important to visualize that the cell always tries to extend its life under regular developmental conditions (the exceptions being programmed cell death that occurs during hypersensitive response to kill invading pathogens, thus killing both the pathogen and the cell/tissue, formation of xylem vessels, secondary xylem tissues, etc.), and the turnover reactions are a part of maintaining the homeostasis. The cell ultimately succumbs to the catabolic reactions during senescence. The compartmentalization and storage of chemical energy in the form of metabolizable macromolecules are all the inherent properties of life, which is defined as a struggle against increasing entropy.

The biosynthesis and catabolism of sucrose is an important part of carbohydrate metabolism. Sucrose is the major form of transport sugar and is translocated through the phloem tissues to other parts of the plant. It is conceivable that photosynthetically fixed carbon from leaf tissues may be transported to the fruits as sucrose during fruit development. Sucrose is biosynthesized from glucose-1-phosphate by three major steps (Fig. 3.3). The first reaction involves the conversion of glucose-1-phosphate to UDP-glucose, by UDP-glucose

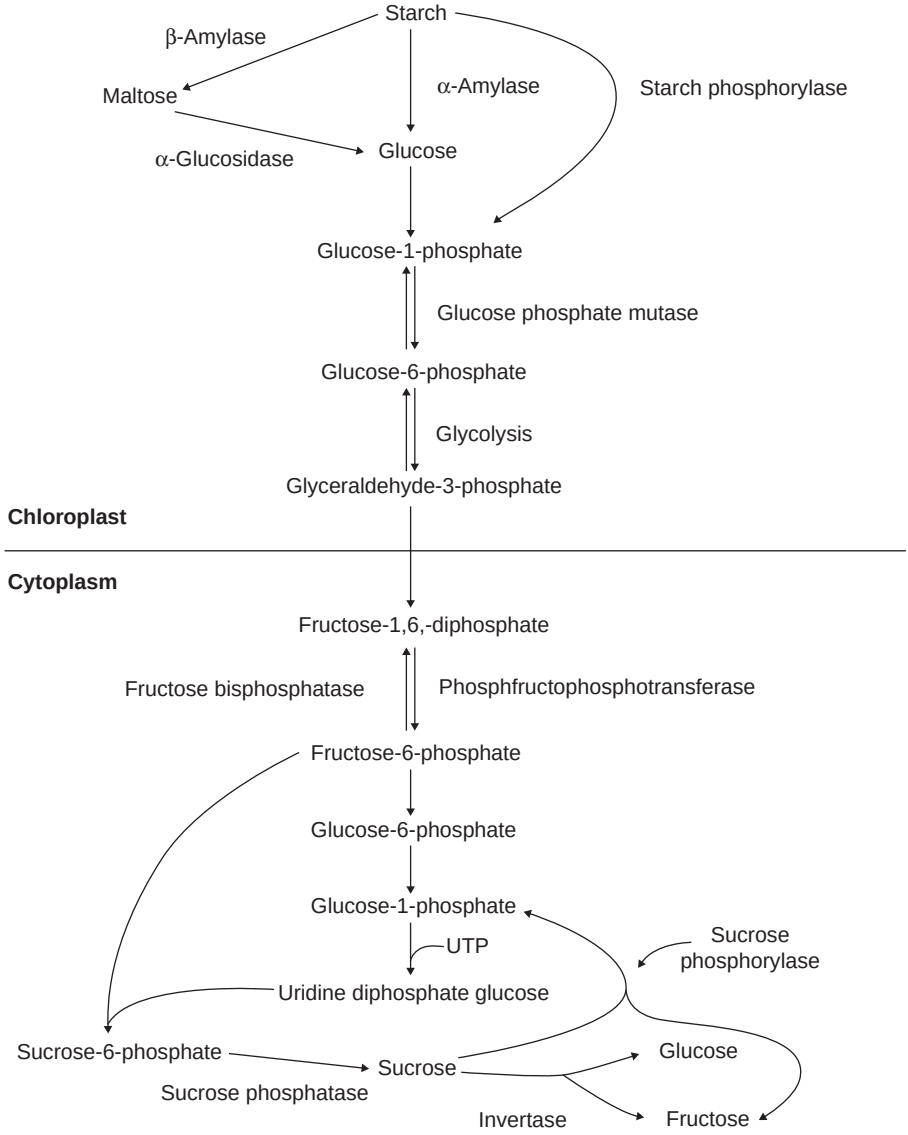


Fig. 3.2 Starch–sugar interconversions in plants and metabolite transfer from chloroplast to the cytoplasm.

pyrophosphorylase in the presence of UTP (uridine triphosphate). UDP-glucose is also an important substrate for the biosynthesis of cell wall components such as cellulose. UDP-glucose is converted to sucrose-6-phosphate by the enzyme sucrose phosphate synthase, which utilizes fructose-6-phosphate during this reaction. Finally, sucrose is formed from sucrose-6-phosphate by the action of phosphatase with the liberation of the inorganic phosphate.

Even though sucrose biosynthesis is an integral part of starch metabolism, sucrose often is not the predominant sugar that accumulates in fruits. Sucrose is further converted into glucose and fructose by the action of invertase, which is characteristic to many ripe fruits.

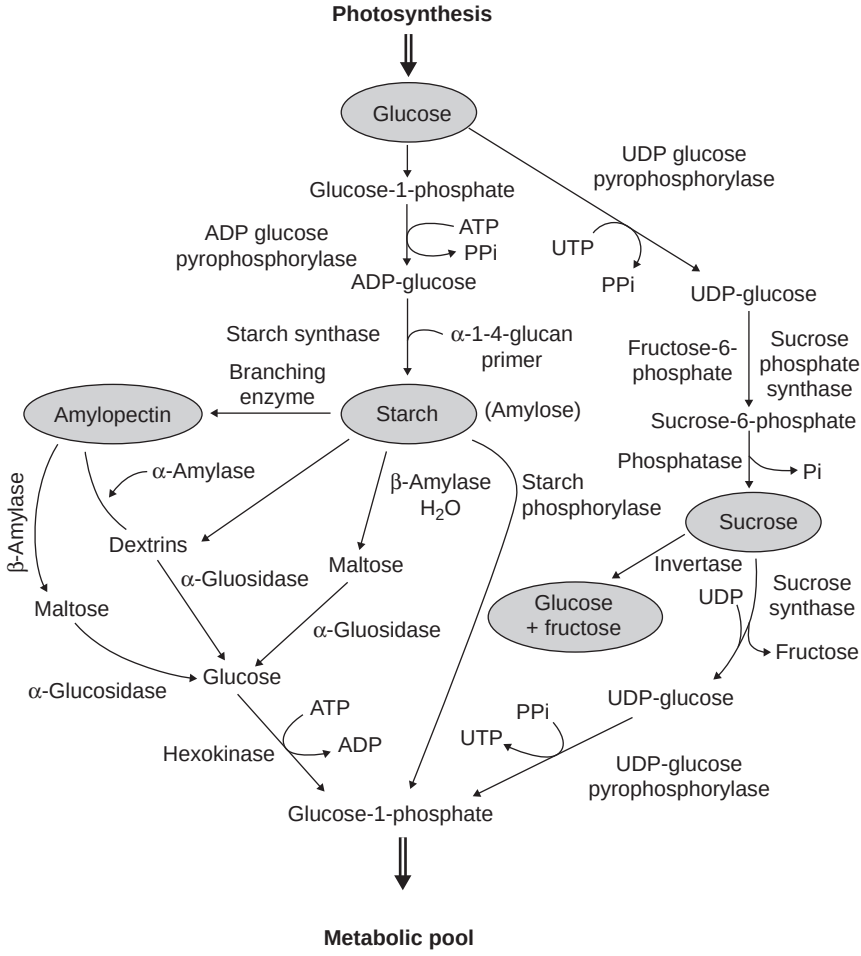


Fig. 3.3 Carbohydrate metabolism in fruits.

Or, by the actions of sucrose synthase and UDP-glucose pyrophosphorylase, glucose-1-phosphate can be regenerated from sucrose. As well, sugar alcohols such as sorbitol and mannitol are major transport and storage components in apple and olive, respectively.

Biosynthesis and catabolism of starch has been extensively studied in banana, where prior to ripening, it can account for 20–25% by fresh weight of the pulp tissue. All the starch-degrading enzymes—α-amylase, β-amylase, α-glucosidase, and starch phosphorylase—have been isolated from banana pulp. The activities of these enzymes increase during ripening. Concomitant with the catabolism of starch, there is an accumulation of the sugars, primarily, sucrose, glucose, and fructose. At the initiation of ripening, sucrose appears to be the major sugar component, which declines during the advancement of ripening with a simultaneous increase in glucose and fructose through the action of invertase (Beaudry et al., 1989). Mango is another fruit that stores large amounts of starch. The starch is degraded by the activities of amylases during the ripening process. In mango, glucose, fructose, and sucrose are the major forms of simple sugars (Selvaraj et al., 1989). The sugar

content is generally very high in ripe mangoes and can reach levels in excess of 90% of the total soluble solids content. Fructose is the predominant sugar in mangoes. In contrast to the bananas, the sucrose levels increase with the advancement of ripening in mangoes, potentially due to gluconeogenesis from organic acids (Kumar and Selvaraj, 1990). As well, the levels of pentose sugars increase during ripening, and could be related to an increase in the activity of the pentose phosphate pathway.

3.3.1.3 Glycolysis

The conversion of starch to sugars and their subsequent metabolism occur in different compartments. During the development of fruits, photosynthetically fixed carbon is utilized for both respiration and biosynthesis. During this phase, the biosynthetic processes dominate. As the fruit matures and begin to ripen, the pattern of sugar utilization changes. Ripening is a highly energy-intensive process. And this is reflected in the burst in respiratory carbon dioxide evolution during ripening. As mentioned earlier, the respiratory burst is characteristic of some fruits that are designated as climacteric fruits. The postharvest shelf life of fruits can depend on their intensity of respiration. Fruits such as mango and banana possess high level of respiratory activity and are highly perishable. The application of controlled atmosphere conditions having low oxygen levels and low temperature have thus become a routine technology for the long-term preservation of fruits.

The sugars and sugar phosphates generated during the catabolism of starch are metabolized through the glycolysis and citric acid cycle (Fig. 3.4). Sugar phosphates can also be channeled through the pentose phosphate pathway, which is a major metabolic cycle that provides reducing power for biosynthetic reactions in the form of NADPH, as well as supplying carbon skeletons for the biosynthesis of several secondary plant products. The organic acids stored in the vacuole are metabolized through the functional reversal of respiratory pathway, which is termed as gluconeogenesis. Altogether, sugar metabolism is a key biochemical characteristic of the fruits.

In the glycolytic steps of reactions (Fig. 3.4), glucose-6-phosphate is isomerized to fructose-6-phosphate by the enzyme hexose phosphate isomerase. Glucose-6-phosphate is derived from glucose-1-phosphate by the action of glucose phosphate mutase. Fructose-6-phosphate is phosphorylated at the C1 position yielding fructose-1,6-bisphosphate. This reaction is catalyzed by the enzyme phosphofructokinase in the presence of ATP. Fructose-1,6-bisphosphate is further cleaved into two three-carbon intermediates, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate, catalyzed by the enzyme aldolase. These two compounds are interconvertible through an isomerization reaction mediated by triose phosphate isomerase. Glyceraldehyde-3-phosphate is subsequently phosphorylated at the C1 position using orthophosphate, as well as oxidized using nicotinamide adenine dinucleotide (NAD), to generate 1,3-diphosphoglycerate and nicotinamide adenine dinucleotide plus hydrogen (NADH). In the next reaction, 1,3-diphosphoglycerate is dephosphorylated by glycerate-3-phosphate kinase in the presence of ADP, along with the formation of ATP. Glycerate-3-phosphate formed during this reaction is further isomerized to 2-phosphoglycerate in the presence of phosphoglycerate mutase. In the presence of the enzyme enolase, 2-phosphoglycerate is converted to phosphoenol pyruvate (PEP). Dephosphorylation of phosphoenolpyruvate in the presence of ADP by pyruvate kinase yields pyruvate and ATP. Metabolic fate of pyruvate is highly regulated. Under normal conditions, it is converted to acetyl coA, which then enters the citric acid cycle. Under anaerobic

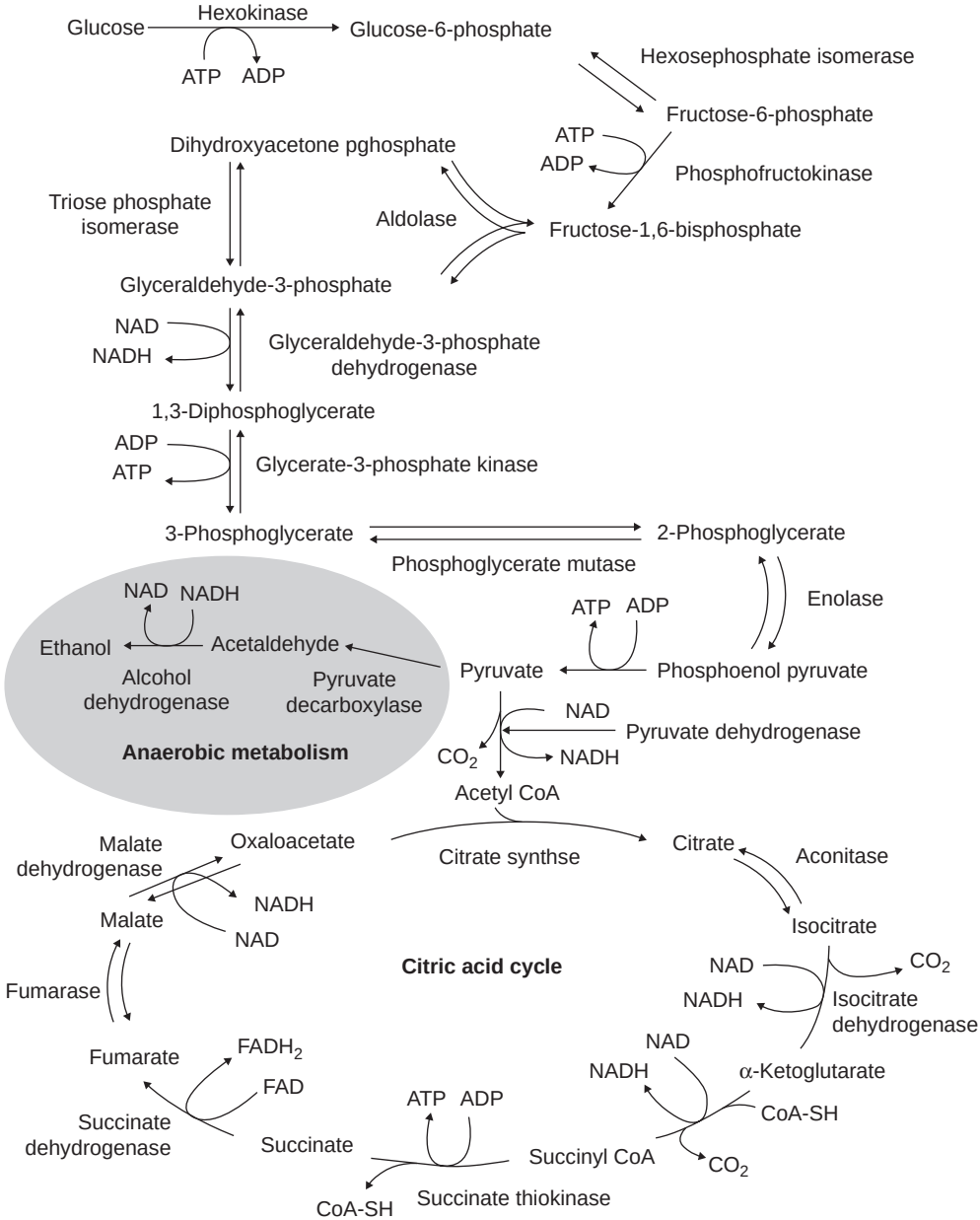


Fig. 3.4 Catabolism of sugars through glycolytic pathway and citric acid cycle.

conditions, pyruvate can be metabolized to ethanol, which is a byproduct in several ripening fruits.

There are two key regulatory steps in glycolysis: one mediated by phosphofructokinase (PFK) and the other by pyruvate kinase. In addition, there are other types of modulation involving cofactors and enzyme structural changes reported to be involved in glycolytic

control. ATP levels increase during ripening. However, in fruits, this does not cause a feedback inhibition of phosphofructokinase as observed in animal systems. There are two isozymes of PFK in plants: one localized in plastids and the other localized in the cytoplasm. These isozymes regulate the flow of carbon from the hexose phosphate pool to the pentose phosphate pool. PFK isozymes are strongly inhibited by phosphoenol pyruvate. Thus, any conditions that may cause the accumulation of phosphoenol pyruvate will tend to reduce the carbon flow through glycolysis. By contrast, inorganic phosphate is a strong activator of PFK. Thus, the ratio of PEP to inorganic phosphate would appear to be the major factor that regulates the activity of PFK and carbon flux through glycolysis. Structural alteration of phosphofructokinase, which increases the efficiency of utilization of fructose-6-phosphate, is another means of regulation that can activate the carbon flow through the glycolytic pathway.

Other enzymes of the glycolytic pathway are involved in the regulation of starch/sucrose biosynthesis (Figs 3.2 and 3.3). Fructose-1,6-bisphosphate is converted back to fructose-6-phosphate by the enzyme fructose-1,6-bisphosphatase, also releasing inorganic phosphate. This enzyme is localized in the cytosol and chloroplast. Fructose-6-phosphate is converted to fructose-2,6-bisphosphate by fructose-6-phosphate 2-kinase, which can be dephosphorylated at the 2-position by fructose-2,6-bisphosphatase. Fructose-6-phosphate is an intermediary in sucrose biosynthesis (Fig. 3.3). Sucrose phosphate synthase (SPS) is regulated by reversible phosphorylation (a form of posttranslational modification that involves addition of a phosphate moiety from ATP to an OH amino acid residue in the protein, such as serine or threonine, mediated by a kinase, and dephosphorylation mediated by a phosphatase) by SPS kinase and SPS phosphatase. Phosphorylation of the enzyme makes it less active. Glucose-6-phosphate is an allosteric activator (a molecule that can bind to an enzyme and increase its activity through enzyme subunit association) of the active form of SPS (dephosphorylated). Glucose-6-phosphate is an inhibitor of SPS kinase, and inorganic phosphate is an inhibitor of SPS phosphatase. Thus, under conditions when glucose-6-phosphate/inorganic phosphate ratio is high, the active form of SPS will dominate, favoring sucrose phosphate biosynthesis. These regulations are highly complex and may be regulated by the flux of other sugars in several pathways.

The conversion of PEP to pyruvate mediated by pyruvate kinase is another key metabolic step in the glycolytic pathway and is irreversible. Pyruvate is used in several metabolic reactions. During respiration, pyruvate is further converted to acetyl coenzyme A (acetyl CoA), which enters the citric acid cycle through which it is completely oxidized to carbon dioxide (Fig. 3.3). The conversion of pyruvate to acetyl CoA is mediated by the enzyme complex pyruvate dehydrogenase and is an oxidative step that involves the formation of NADH from NAD. Acetyl CoA is a key metabolite and starting point for several biosynthetic reactions (fatty acids, isoprenoids, phenylpropanoids, etc.).

3.3.1.4 Citric acid cycle

The citric acid cycle involves the biosynthesis of several organic acids, many of which serve as precursors for the biosynthesis of several groups of amino acids. In the first reaction, oxaloacetate combines with acetyl CoA to form citrate and is mediated by citrate synthase (Fig. 3.4). In the next step, citrate is converted to isocitrate by the action of aconitase. The next two steps in the cycle involve oxidative decarboxylation. The conversion of isocitrate to α-ketoglutarate involves the removal of a carbon dioxide molecule and reduction of

NAD to NADH. This step is catalyzed by isocitrate dehydrogenase. α-Ketoglutarate is converted to succinyl CoA by α-ketoglutarate dehydrogenase, along with the removal of another molecule of carbon dioxide and the conversion of NAD to NADH. Succinate, the next product, is formed from succinyl CoA by the action of succinyl CoA synthetase that involves the removal of the CoA moiety and the conversion of ADP to ATP. Through these steps, the complete oxidation of the acetyl CoA moiety has been achieved with the removal of two molecules of carbon dioxide. Thus, succinate is a four-carbon organic acid. Succinate is further converted to fumarate and malate in the presence of succinate dehydrogenase and fumarase, respectively. Malate is oxidized to oxaloacetate by the enzyme malate dehydrogenase along with the conversion of NAD to NADH. Oxaloacetate then can combine with another molecule of acetyl CoA to repeat the cycle. The reducing power generated in the form of NADH and FADH (succinate dehydrogenation step) is used for the biosynthesis of ATP through the transport of electrons through the electron transport chain in the mitochondria.

3.3.1.5 Gluconeogenesis

Several fruits store large amounts of organic acids in their vacuole, and these acids are converted back to sugars during ripening, a process termed as gluconeogenesis. Several irreversible steps in the glycolysis and citric acid cycle are bypassed during gluconeogenesis. Malate and citrate are the major organic acids present in fruits. In fruits such as grapes, where there is a transition from a sour to a sweet stage during ripening, organic acids content declines. Grape contains predominantly tartaric acid along with malate, citrate, succinate, fumarate, and several organic acid intermediates of metabolism. The content of organic acids in berries can affect their suitability for processing. High acid content coupled with low sugar content can result in poor quality wines. External warm growth conditions enhance the metabolism of malic acid in grapes during ripening and could result in a high tartarate/malate ratio, which is considered ideal for vinification.

The metabolism of malate during ripening is mediated by the malic enzyme, NADP-dependent malate dehydrogenase. Along with a decline in malate content, there is a concomitant increase in the sugars suggesting a possible metabolic precursor product relationship between these two events. Indeed, when grape berries were fed with radiolabeled malate, the radiolabel could be recovered in glucose. The metabolism of malate involves its conversion to oxaloacetate mediated by malate dehydrogenase, the decarboxylation of oxaloacetate to phosphoenol pyruvate catalyzed by PEP-carboxykinase, and a reversal of glycolytic pathway leading to sugar formation (Ruffner et al., 1983). The gluconeogenic pathway from malate may contribute only a small percentage (5%) of the sugars, and a decrease in malate content could primarily result from reduced synthesis and increased catabolism through the citric acid cycle. The inhibition of malate synthesis by the inhibition of the glycolytic pathway could result in increased sugar accumulation. Metabolism of malate in apple fruits is catalyzed by NADP-malic enzyme that converts malate to pyruvate. In apples, malate appears to be primarily oxidized through the citric acid cycle. Organic acids are important components of citrus fruits. Citric acid is the major form of the acid followed by malic acid and several less abundant acids such as acetate, pyruvate, oxalate, glutarate, and fumarate. In oranges, the acidity increases during maturation of the fruit and declines during the ripening phase. Lemon fruits, by contrast, increase their acid content through the accumulation of citrate. The citrate levels in various citrus fruits range from 75 to 88%, and malate levels

range from 2 to 20%. Ascorbate is another major component of citrus fruits. Ascorbate levels can range from 20 to 60 mg/100 g juice in various citrus fruits. The orange skin may possess 150–340 mg/100 g fresh weight of ascorbate, which may not be extracted into the juice.

3.3.1.6 Anaerobic respiration

Anaerobic respiration is a common event in the respiration of ripe fruits and especially becomes significant when fruits are exposed to low temperature. Often, this may result from oxygen-depriving conditions induced inside the fruit. Under anoxia, ATP production through the citric acid cycle and mitochondrial electron transport chain is inhibited. Anaerobic respiration is a means of regenerating NAD, which can drive the glycolyic pathway and produce minimal amounts of ATP (Fig. 3.4). Under anoxia, pyruvate formed through glycolysis is converted to lactate by lactate dehydrogenase using NADH as the reducing factor, and generating NAD. Accumulation of lactate in the cytosol could cause acidification, and under these low pH conditions, lactate dehydrogenase is inhibited. The formation of acetaldehyde by the decarboxylation of pyruvate is stimulated by the activation of pyruvate decarboxylase under low pH conditions in the cytosol. It is also likely that the increase in concentration of pyruvate in the cytoplasm may stimulate pyruvate decarboxylase directly. Acetaldehyde is reduced to ethanol by alcohol dehydrogenase using NADH as the reducing power. Thus, acetaldehyde and ethanol are common volatile components observed in the headspace of fruits, indicative of the occurrence of anaerobic respiration. Cytosolic acidification is a condition that stimulates deteriorative reactions. By removing lactate through efflux and converting pyruvate to ethanol, cytosolic acidification can be avoided.

Anaerobic respiration plays a significant role in the respiration of citrus fruits. During early stages of growth, respiratory activity predominantly occurs in the skin tissue. Oxygen uptake by the skin tissue was much higher than the juice vesicles (Purvis, 1985). With advancing maturity, a decline in aerobic respiration and an increase in anaerobic respiration was observed in Hamlin orange skin (Bruemmer, 1989). In parallel with this, the levels of ethanol and acetaldehyde increased. As well, a decrease in the organic acid substrates, pyruvate and oxaloacetate, was detectable in Hamlin orange juice. An increase in the activity levels of pyruvate decarboxylase, alcohol dehydrogenase, and malic enzyme was noticed in parallel with the decline in pyruvate and accumulation of ethanol. In apple fruits, malic acid is converted to pyruvate by the action of NADP-malic enzyme, and pyruvate subsequently converted to ethanol by the action of pyruvate decarboxylase and alcohol dehydrogenase. The alcohol dehydrogenase in apple can use NADPH as a cofactor, and NADP is regenerated during ethanol production, thus driving malate utilization. Ethanol is either released as a volatile or can be used for the biosynthesis of ethyl esters of volatiles.

3.3.1.7 Pentose phosphate pathway

Oxidative pentose phosphate pathway (PPP) is a key metabolic pathway that provides reducing power (NADPH) for biosynthetic reactions as well as carbon precursors for the biosynthesis of amino acids, nucleic acids, secondary plant products, etc. The PPP shares many of the sugar phosphate intermediates with the glycolytic pathway (Fig. 3.5). The PPP is characterized by the interconversion of sugar phosphates with three (glyceraldehyde-3-phosphate), four (erythrose-4-phosphate), five (ribulose, ribose, xylulose phosphates),

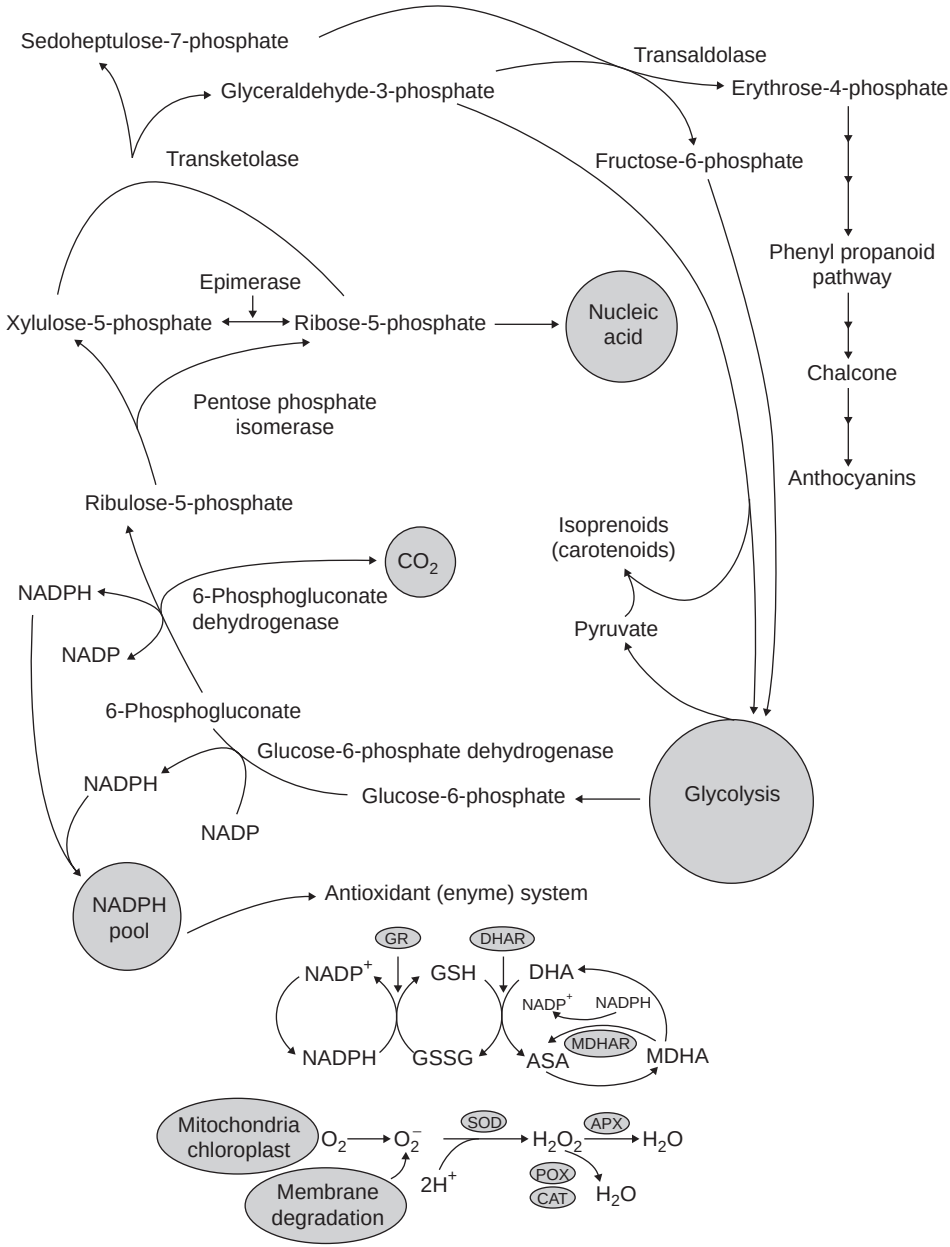


Fig. 3.5 Oxidative pentose phosphate pathway in plants. NADPH generated from the pentose phosphate pathway is channeled into the antioxidant enzyme system where the regeneration of oxidized intermediates requires NADPH. GSH, reduced glutathione; GSSG, oxidized glutathione; ASA, reduced ascorbate; MDHA, monodehydroascorbate; DHA, dehydroascorbate; GR, glutathione reductase; DHAR, dehydroascorbate reductase; MDHAR, monodehydroascorbate reductase; SOD, superoxide dismutase; CAT, catalase; POX, peroxidase; APX, ascorbate peroxidase.

six (glucose-6-phosphate, fructose-6-phosphate), and seven (sedoheptulose-7-phosphate) carbons.

PPP involves the oxidation of glucose-6-phosphate, and the sugar phosphate intermediates formed are recycled. The first two reactions of PPP are oxidative reactions mediated by the enzymes glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Fig. 3.5). In the first step, glucose-6-phosphate is converted to 6-phosphogluconate by the removal of two hydrogen atoms by NADP to form NADPH. In the next step, 6-phosphogluconate, a six-carbon sugar acid phosphate, is converted to ribulose-5-phosphate, a five-carbon sugar phosphate. This reaction involves the removal of a carbon dioxide molecule along with the formation of NADPH. Ribulose-5-phosphate undergoes several metabolic conversions to yield fructose-6-phosphate. Fructose-6-phosphate can then be converted back to glucose-6-phosphate by the enzyme glucose-6-phosphate isomerase and the cycle repeated. Thus, six complete turns of the cycle can result in the complete oxidation of a glucose molecule.

Despite the differences in the reaction sequences, the glycolytic pathway and the PPP intermediates can interact with one another and share common intermediates. Intermediates of both the pathways are localized in plastids as well as the cytoplasm, and intermediates can be transferred across the plastid membrane into the cytoplasm and back into the chloroplast. Glucose-6-phosphate dehydrogenase is localized in the both chloroplast and cytoplasm. Cytosolic glucose-6-phosphate dehydrogenase activity is strongly inhibited by NADPH. Thus, the ratio of NADP to NADPH could be the regulatory control point for the enzyme function. The chloroplastic enzyme is regulated differently through oxidation and reduction, and related to the photosynthetic process. 6-Phosphogluconate dehydrogenase exists as distinct cytosol- and plastid-localized isozymes.

PPP is a key metabolic pathway related to biosynthetic reactions, antioxidant enzyme function, and general stress tolerance of the fruits. Ribose-5-phosphate is used in the biosynthesis of nucleic acids, and erythrose-4-phosphate is channeled into phenyl propanoid pathway leading to the biosynthesis of the amino acids phenylalanine and tryptophan. Phenylalanine is the metabolic starting point for the biosynthesis of flavonoids and anthocyanins in fruits. Glyceraldehyde-3-phosphate and pyruvate serve as the starting intermediates for the isoprenoid pathway localized in the chloroplast. Accumulation of sugars in fruits during ripening has been related to the function of PPP. In mangoes, increase in the levels of pentose sugars observed during ripening has been related to increased activity of PPP. Increases in glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities were observed during ripening of mango.

NADPH is a key component required for the proper functioning of the antioxidant enzyme system (Fig. 3.5). During growth, stress conditions, fruit ripening, and senescence, free radicals are generated within the cell. Activated forms of oxygen, such as superoxide, hydroxyl, and peroxy radicals can attack enzymes and proteins, nucleic acids, lipids in the biomembrane, etc., causing structural and functional alterations of these molecules. Under most conditions, these are deleterious changes, which are nullified by the action of antioxidants and antioxidant enzymes. Simple antioxidants such as ascorbate and vitamin E can scavenge the free radicals and protect the tissue. Anthocyanins and other polyphenols may also serve as simple antioxidants. In addition, the antioxidant enzyme system involves the integrated function of several enzymes. The key antioxidant enzymes are superoxide dismutase (SOD), catalase, ascorbate peroxidase, and peroxidase. SOD converts superoxide into

hydrogen peroxide. Hydrogen peroxide is immediately acted upon by catalase, generating water. Hydrogen peroxide can also be removed by the action of peroxidases. A peroxidase uses the oxidation of a substrate molecule (usually having a phenol structure, C—OH, which becomes a quinone, C═O, after the reaction) to react with hydrogen peroxide, converting it to water. Hydrogen peroxide can also be acted upon by ascorbate peroxidase, which uses ascorbate as the hydrogen donor for the reaction, resulting in water formation. The oxidized ascorbate is regenerated by the action of a series of enzymes (Fig. 3.5). These include monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR). Dehydroascorbate is reduced to ascorbate using reduced glutathione (GSH) as a substrate, which itself gets oxidized (GSSG) during this reaction. The oxidized glutathione is reduced back to GSH by the activity of glutathione reductase using NADPH. Antioxidant enzymes exist as several functional isozymes with differing activities and kinetic properties in the same tissue. These enzymes are also compartmentalized in chloroplast, mitochondria, and cytoplasm. The functioning of the antioxidant enzyme system is crucial to the maintenance of fruit quality through preserving cellular structure and function (Meir and Bramlage, 1988; Ahn et al., 1992).

3.3.2 Lipid metabolism

Among fruits, avocado and olive are the only fruits that significantly store reserves in the form of lipid triglycerides. In avocado, triglycerides form the major part of the neutral lipid fraction, which can account for nearly 95% of the total lipids. Palmitic (16:0), palmitoleic (16:1), oleic (18:1), and linoleic (18:2) acids are the major fatty acids of triglycerides. The oil content progressively increases during maturation of the fruit, and the oils are compartmentalized in oil bodies or oleosomes. The biosynthesis of fatty acids occurs in the plastids, and the fatty acids are exported into the endoplasmic reticulum where they are esterified with glycerol-3-phosphate by the action of a number of enzymes to form the triglyceride. The triglyceride-enriched regions then are believed to bud off from the endoplasmic reticulum as the oil body. The oil body membranes are different from other cellular membranes since they are made up of only a single layer of phospholipids. The triglycerides are catabolized by the action of triacylglycerol lipases, which release the fatty acids. The fatty acids are then broken down into acety CoA units through β-oxidation.

Even though phospholipids constitute a small fraction of the lipids in fruits, the degradation of phospholipids is a key factor that controls the progression of senescence. As in several senescing systems, there is a decline in phospholipids as the fruit undergoes senescence. With the decline in phospholipids content, there is a progressive increase in the levels of neutral lipids, primarily diacylglycerols, free fatty acids, and fatty aldehydes. In addition, the levels of sterols may also increase. Thus, there is an increase in the ratio of sterol/phospholipids. Such changes in the composition of membrane can cause the formation of gel-phase or nonbilayer lipid structures (micelles). These changes can make the membranes leaky, thus resulting in the loss of compartmentalization, and ultimately, senescence (Paliyath and Droillard, 1992).

Membrane lipid degradation occurs by the tandem action of several enzymes, one enzyme acting on the product released by the previous enzyme in the sequence. Phospholipase D (PLD) is the first enzyme of the pathway, which initiates phospholipids catabolism,

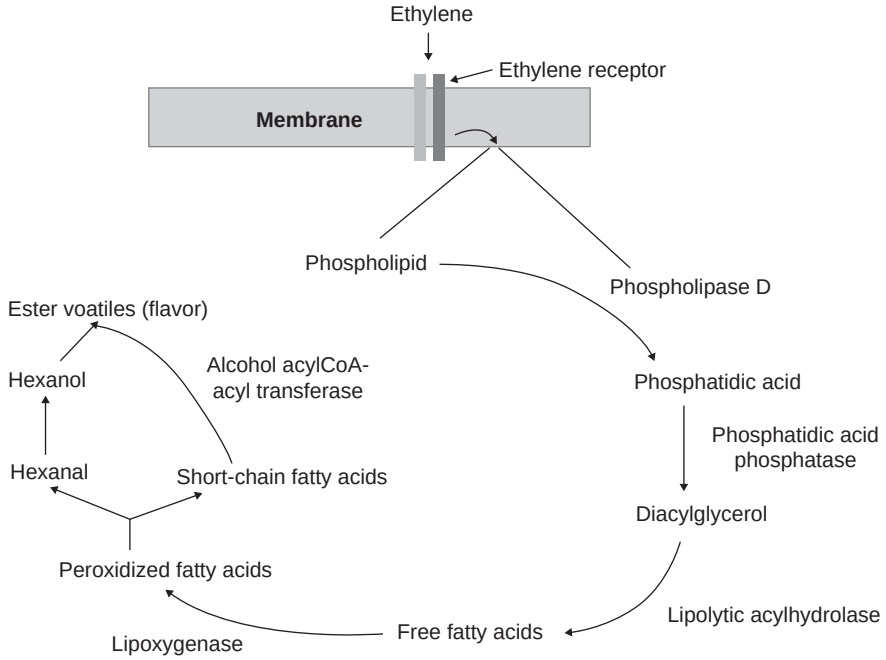


Fig. 3.6 Phospholipid catabolic pathway and its relation to fruit ripening.

and is a key enzyme of the pathway (Fig. 3.6). Phospholipase D acts on phospholipids, liberating phosphatidic acid and the respective headgroup (choline, ethanolamine, glycerol, inositol). Phosphatidic acid in turn is acted upon by phosphatidate phosphatase, which removes the phosphate group from phosphatidic acid, with the liberation of diacylglycerols (diglycerides). The acyl chains of diacylglycerols are then deesterified by the enzyme lipolytic acyl hydrolase, liberating free fatty acids. Unsaturated fatty acids with a *cis*-1,4-pentadiene structure (linoleic acid, linolenic acid) are acted upon by lipoxygenase, causing the peroxidation of fatty acids. This step may also cause the production of activated oxygen species such as singlet oxygen, superoxide, and peroxy radicals. The peroxidation products of linolenic acid can be 9-hydroperoxy linoleic acid or 13-hydroperoxy linoleic acid. The hydroperoxylinoleic acids undergo cleavage by hydroperoxide lyase resulting in several products including hexanal, hexenal, and ω-keto fatty acids (keto group toward the methyl end of the molecule). For example, hydroperoxide lyase action on 13-hydroperoxylinolenic acid results in the formation of *cis*-3-hexenal and 12-keto-*cis*-9-dodecenoic acid. Hexanal and hexenal are important fruit volatiles. The short-chain fatty acids may feed into catabolic pathway (β-oxidation) that results in the formation of short-chain acyl CoAs, ranging from acetyl CoA to dodecanoyl CoA. The short-chain acyl CoAs and alcohols (ethanol, propanol, butanol, pentanol, hexanol, etc.) are esterified to form a variety of esters that constitute components of flavor volatiles that are characteristic to fruits. The free fatty acids and their catabolites (fatty aldehydes, fatty alcohols, alkanes, etc.) can accumulate in the membrane, causing membrane destabilization (formation of gel-phase, nonbilayer structures, etc.). An interesting regulatory feature of this pathway is the very low substrate specifity of enzymes

that act downstream from phospholipase D, for the phospholipids. Thus, phosphatidate phosphatase, lipolytic acyl hydrolase, and lipoxygenase do not directly act on phospholipids, though there are exceptions to this rule. Therefore, the degree of membrane lipid catabolism will be determined by the extent of activation of phospholipase D.

The membrane lipid catabolic pathway is considered as an autocatalytic pathway. The destabilization of the membrane can cause the leakage of calcium and hydrogen ions from the cell wall space, as well as the inhibition of calcium and proton ATPases, the enzymes responsible for maintaining a physiological calcium and proton concentration within the cytoplasm (calcium concentration below micromolar range, pH in the 6–6.5 range). Under conditions of normal growth and development, these enzymes pump the extra calcium and hydrogen ions that enter the cytoplasm from storage areas such as apoplast and the ER lumen, in response to hormonal and environmental stimulation using ATP as the energy source. The activities of calcium and proton ATPases localized on plasma membrane, endoplasmic reticulum, and the tonoplast are responsible for pumping the ions back into the storage areas. In fruits (and other senescing systems), with the advancement in ripening and senescence, there is a progressive increase in leakage of calcium and hydrogen ions. Phospholipase D is stimulated by low pH and calcium concentration over 10 μM. Thus, if the cytosolic concentrations of these ions progressively increase during ripening or senescence, the membranes are damaged as a consequence. However, this is an inherent feature of the ripening process in fruits, which results in the development of ideal organoleptic qualities that makes them edible. The uncontrolled membrane deterioration can result in the loss of shelf life and quality in fruits.

The properties and regulation of the membrane degradation pathway are increasingly becoming clear. Enzymes such as phospholipase D (PLD) and lipoxygenase (LOX) are very well studied. There are several isoforms of phospholipase D designated as PLD-α, PLD-β, PLD-γ, etc. The expression and activity levels of PLD-α are much higher than that of the other PLD isoforms. Thus, PLD-α is considered as a housekeeping enzyme. The regulation of PLD activity is an interesting feature. PLD is normally a soluble enzyme. The secondary structure of PLD shows the presence of a segment of around 130 amino acids at the N-terminal end, designated as the C2 domain. This domain is characteristic of several enzymes and proteins that are integral components of the hormone signal transduction system. In response to hormonal and environmental stimulation and the resulting increase in cytosolic calcium concentration, C2 domain binds calcium and transports PLD to the membrane where it can initiate membrane lipid degradation. The precise relation between the stimulation of the ethylene receptor and phospholipase D activation is not fully understood, but could involve the release of calcium and migration of PLD to the membrane. PLD-α appear to be the key enzyme responsible for the initiation of membrane lipid degradation in tomato fruits. Antisense inhibition of PLD-α in tomato fruits resulted in the reduction of PLD activity and, consequently, an improvement in the shelf life, firmness, soluble solids, and lycopene content of the ripe fruits (Oke et al., 2003; Pinhero et al., 2003). There are other phospholipid-degrading enzymes such as phospholipase C and phospholipase A_2. Several roles of these enzymes in signal transduction processes have been extensively reviewed (Wang, 2001; Meijer and Munnik, 2003).

Lipoxygenase exists as both soluble and membranous forms in tomato fruits (Todd et al., 1990). Very little information is available on phosphatidate phosphatase and lipolytic acyl hydrolase in fruits.

3.3.3 Proteolysis and structure breakdown in chloroplasts

The major proteinaceous compartments in fruits are the chloroplasts that are distributed in the epidermal and hypodermal layers of fruits. The chloroplasts are not very abundant in fruits. During senescence, the chloroplast structure is gradually disassembled with a decline in chlorophyll levels due to degradation and disorganization of the grana lamellar stacks of the chloroplast. With the disorganization of the thylakoid, globular structures termed as plastoglobuli accumulate within the chloroplast stroma and are rich in degraded lipids. The degradation of chloroplasts and chlorophyll results in the unmasking of other colored pigments and is a prelude to the state of ripening and development of organoleptic qualities. Mitochondria, which are also rich in protein, are relatively stable and undergo disassembly during the latter part of ripening and senescence.

Chlorophyll degradation is initiated by the enzyme chlorophyllase that splits chlorophyll into chlorophyllide and the phytol chain. Phytol chain is made up of isoprenoid units (methyl-1,3-butadiene) and its degradation products accumulate in the plastoglobuli. Flavor components such as 6-methyl-5-heptene-2-one, a characteristic component of tomato flavor, are also produced by the catabolism of phytol chain. The removal of magnesium from chlorophyllide results in the formation of pheophorbide. Pheophorbide, which possesses a tetrapyrole structure, is converted to a straight-chain colorless tetrapyrrole by the action of pheophorbide oxidase. Action of several other enzymes is necessary for the full catabolism of chlorophyll. The protein complexes that organize the chlorophyll, the light-harvesting complexes, are degraded by the action of several proteases. The enzyme ribulose-bis-phosphate carboxylase/oxygenase (Rubisco), the key enzyme in photosynthetic carbon fixation, is the most abundant protein in chloroplast. Rubisco levels also decline during ripening/senescence due to proteolysis. The amino acids resulting from the catabolism of proteins may be translocated to regions where they are needed for biosynthesis. In fruits, they may just enrich the soluble fraction with amino acids.

3.4 Secondary plant products

Secondary plant products are regarded as metabolites that are derived from primary metabolic intermediates through well-defined biosynthetic pathways. The importance of the secondary plant products to the plant or organ in question may not readily be obvious, but these compounds appear to have a role in the interaction of the plant with the environment. The secondary plant products may include nonprotein amino acids, alkaloids, isoprenoid components (terpenes, carotenoids, etc.), flavonoids and anthocyanins, ester volatiles, and several other organic compounds with diverse structure. The number and types of secondary plant products are enormous, but, with the perspective of fruit quality, the important secondary plant products include isoprenoids, anthocyanins, and ester volatiles.

3.4.1 Isoprenoid biosynthesis

In general, isoprenoids possess a basic five-carbon skeleton in the form of 2-methyl-1, 3-butadiene (isoprene), which undergoes condensation to form larger molecules. There are two distinct pathways for the formation of isoprenoids: the acetate/mevalonate pathway

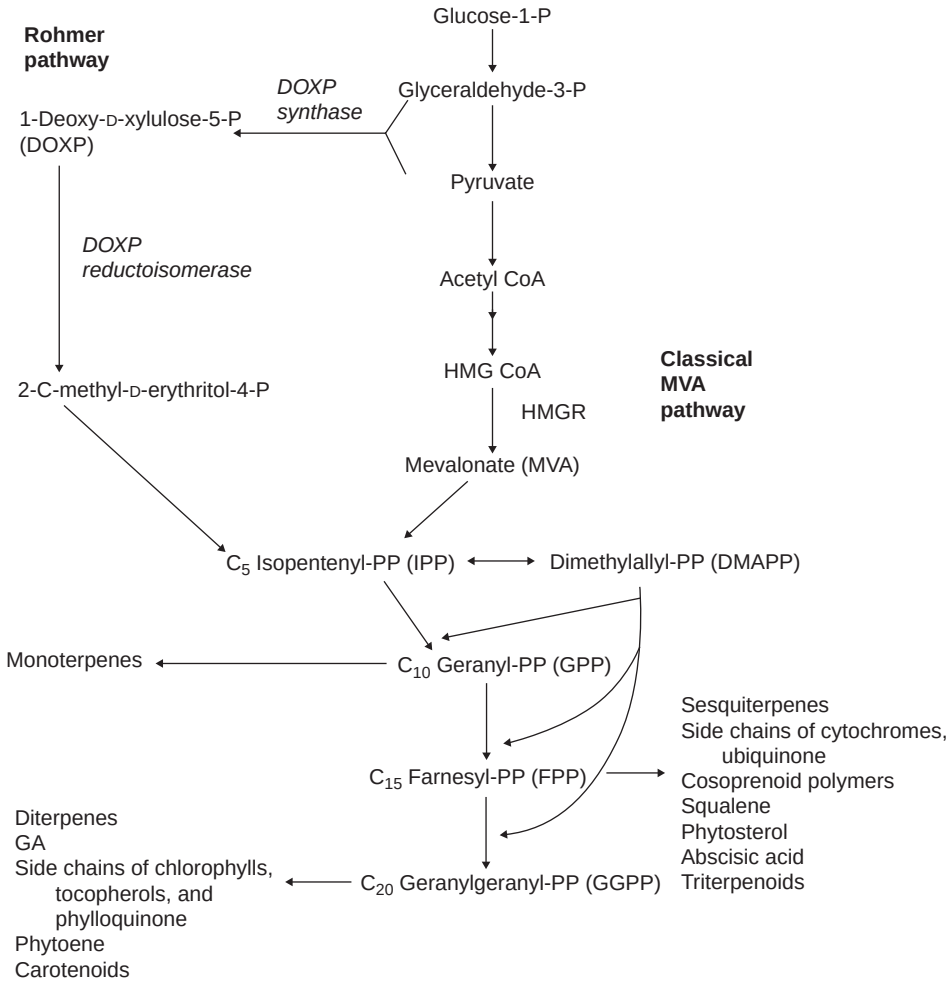


Fig. 3.7 Isoprenoid biosynthetic pathway in plants.

(Bach et al., 1999) localized in the cytosol and the DOXP pathway (Rohmer pathway; Rohmer et al., 1993) localized in the chloroplast (Fig. 3.7). The metabolic precursor for the acetate/mevalonate pathway is acetyl coenzyme A. Through the condensation of three acetyl CoA molecules, a key component of the pathway, 3-hydroxy-3-methyl-glutaryl CoA (HMG CoA), is generated. HMG-CoA undergoes reduction in the presence of NADPH mediated by the key regulatory enzyme of the pathway HMG CoA reductase (HMGR) to form mevalonate. Mevalonate undergoes a two-step phosphorylation in the presence of ATP, mediated by kinases, to form isopentenyl pyrophosphate (IPP), the basic five-carbon condensational unit of several terpenes. IPP is isomerized to dimethylallylpyrophosphate (DMAPP) mediated by the enzyme IPP isomerase. Condensation of these two components results in the synthesis of C10 (geranyl), C15 (farnesyl), and C20 (geranylgeranyl) pyrophosphates. The C10 pyrophosphates give rise to monoterpenes, C15 pyrophosphates give rise to sesquiterpenes, and C20 pyrophosphates give rise to diterpenes. Monoterpenes are major volatile

components of fruits. In citrus fruits, these include components such as limonene, myrcene, and pinene occurring in various proportions. Derivatives of monoterpenes such as geranial, neral (aldehydes), geraniol, linalool, terpineol (alcohols), geranyl acetate, and neryl acetate (esters) are also ingredients of the volatiles of citrus fruits. Citrus fruits are especially rich in monoterpenes and derivatives. α-Farnesene is a major sesquiterpene (C15) component evolved by apples. The catabolism of α-farnesene in the presence of oxygen into oxidized forms has been implicated as a causative feature in the development of the physiological disorder superficial scald (a type of superficial browning) in certain varieties of apples such as red Delicious, McIntosh, and Cortland (Rupasinghe et al., 2000, 2003).

HMGR is a highly conserved enzyme in plants and is encoded by a multigene family (Lichtenthaler et al., 1997). The HMGR genes (*hmg1*, *hmg2*, *hmg3*, etc.) are nuclear encoded and can be differentiated from each other by the sequence differences at the 3′-untranslated regions of the cDNAs. There are three distinct genes for HMGR in tomato, and two in apples. The different HMGR end products may be localized in different cellular compartments and are synthesized differentially in response to hormones, environmental signals, pathogen infection, etc. In tomato fruits, the level of *hmg1* expression is high during early stage of fruit development when cell division and expansion processes are rapid and require high levels of sterols for incorporation into the expanding membrane compartments. The expression of *hmg2*, which is not detectable in young fruits, increases during the latter part of fruit maturation and ripening.

HMGR activity can be detected in both membranous and cytosolic fractions of apple fruit skin tissue extract. HMGR is a membrane-localized enzyme, and the activity is detectable in the endoplasmic reticulum, plastid, and mitochondrial membranes. It is likely that HMGR may have undergone proteolytic cleavage, releasing a fragment into the cytosol, which also possesses enzyme activity. There is a considerable degree of interaction between the different enzymes responsible for the biosynthesis of isoprenoids, which may exist as multienzyme complexes referred to as metabolons. The enzyme farnesyl pyrophosphate synthase, responsible for the synthesis of farnesyl pyrophosphate, is a cytosolic enzyme. Similarly, farnesene synthase, the enzyme that converts farnesyl pyrophosphate to α-farnesene in apples, is a cytosolic enzyme. Thus, several enzymes may act in concert at the cytoplasm/endoplasmic reticulum boundary to synthesize isoprenoids.

HMG CoA reductase expression and activities in apple fruits are hormonally regulated (Rupasinghe et al., 2001, 2003). There are two genes for HMGR in apples designated as *hmg1* and *hmg2*, which are differentially expressed during storage. The expression of *hmg1* was constitutive and the transcripts (mRNA) were present throughout the storage period. By contrast, the expression of *hmg2* increased during storage in parallel with the accumulation of α-farnesene. Ethylene production also increased during storage. Ethylene stimulates the biosynthesis of α-farnesene as evident from the inhibition of α-farnesene biosynthesis and the expression of *hmg2* by the ethylene action inhibitor 1-methylcyclopropene. Thus, biosynthesis of isoprenoids is a highly controlled process.

Carotenoids, which are major isoprenoid components of chloroplasts, are biosynthesized through the Rohmer pathway. The precursors of this pathway are pyruvate and glyceraldehyde-3-phosphate, and through a number of enzymatic steps, 1-deoxy-D-xylulose-5-phosphate (DOXP), a key metabolite of the pathway is formed. NADPH-mediated reduction of DOXP leads ultimately to the formation of IPP. Subsequent condensations of IPP and DMAPP are similar as in the classical mevalonate pathway. Carotenoids

have a stabilizing role in the photosynthetic reactions. By virtue of their structure, they can accept and stabilize excess energy absorbed by the light-harvesting complex. During the early stages of fruit development, the carotenoids have primarily photosynthetic function. As the fruit ripens, the composition of carotenoids changes to reveal the colored xanthophylls pigments. In tomato, lycopene is the major carotenoid pigment that accumulates during ripening. Lycopene is an intermediate of the carotene biosynthetic pathway. In young fruits, lycopene formed by the condensation of two geranylgeranyl pyrophosphate (C20) moieties, mediated by the enzyme phytoene synthase, is converted to β-carotene by the action of the enzyme sesquiterpene cyclase. However, as ripening proceeds, the levels and activity of sesquiterpene cyclase are reduced, leading to the accumulation of lycopene in the stroma. This leads to the development of red color in ripe tomato fruits. In yellow tomatoes, the carotene biosynthesis is not inhibited, and as the fruit ripens, the chlorophyll pigments are degraded exposing the yellow carotenoids. Carotenoids are also major components that contribute to the color of melons. β-Carotene is the major pigment in melons with an orange flesh. In addition, the contribution to color is also provided by α-carotene, δ-carotene, phytofluene, phytoene, lutein, and violaxanthin. In red-fleshed melons, lycopene is the major ingredient, whereas in yellow-fleshed melons, xanthophylls and β-carotene predominate. Carotenoids provide not only a variety of color to the fruits, but are also important nutritional ingredients in human diet. β-Carotene is converted to vitamin A in the human body and thus serves as a precursor to vitamin A. Carotenoids are strong antioxidants. Lycopene is observed to provide protection from cardiovascular diseases and cancer (Giovanucci, 1999). Lutein, a xanthophyll, has been proposed to play a protective role in the retina, maintaining the vision.

3.4.2 Anthocyanin biosynthesis

The development of color is a characteristic feature of the ripening process, and in several fruits, the color components are anthocyanins biosynthesized from metabolic precursors. The anthocyanins accumulate in the vacuole of the cell and are often abundant in the cells closer to the surface of the fruit. Anthocyanin biosynthesis starts by the condensation of three molecules of malonyl CoA with *p*-coumaroyl CoA to form tetrahydroxychalcone, mediated by the enzyme chalcone synthase (Fig. 3.8). Tetrahydroxychalcone has the basic flavonoid structure C6–C3–C6, with two phenyl groups separated by a three-carbon link. Chalcone isomerase enables the ring closure of chalcone leading to the formation of the flavanone naringenin that possesses a flavonoid structure having two phenyl groups linked together by a heterocyclic ring (Fig. 3.9). The phenyl groups are designated as A and B, and the heterocyclic ring is designated as ring C. Subsequent conversions of naringenin by flavonol hydroxylases result in the formation of dihydrokaempferol, dihydromyricetin, and dihydroquercetin, which differ in their number of hydroxyl moieties. Dihydroflavonol reductase converts the dihydroflavonols into the colorless anthocyanidin compounds leucocyanidin, leucopelargonidin, and leucodelphinidin. Removal of hydrogens and the induction of unsaturation of the C ring at C2 and C3, mediated by anthocyanin synthase, results in the formation of cyanidin, pelargonidin, and delphinidin, the colored compounds (Fig. 3.9). Glycosylation, methylation, coumaroylation, and a variety of other additions of the anthocyanidins result in color stabilization of the diverse types of anthocyanins seen in fruits. Pelargonidins give orange, pink, and red color, cyanidins provide magenta and crimson

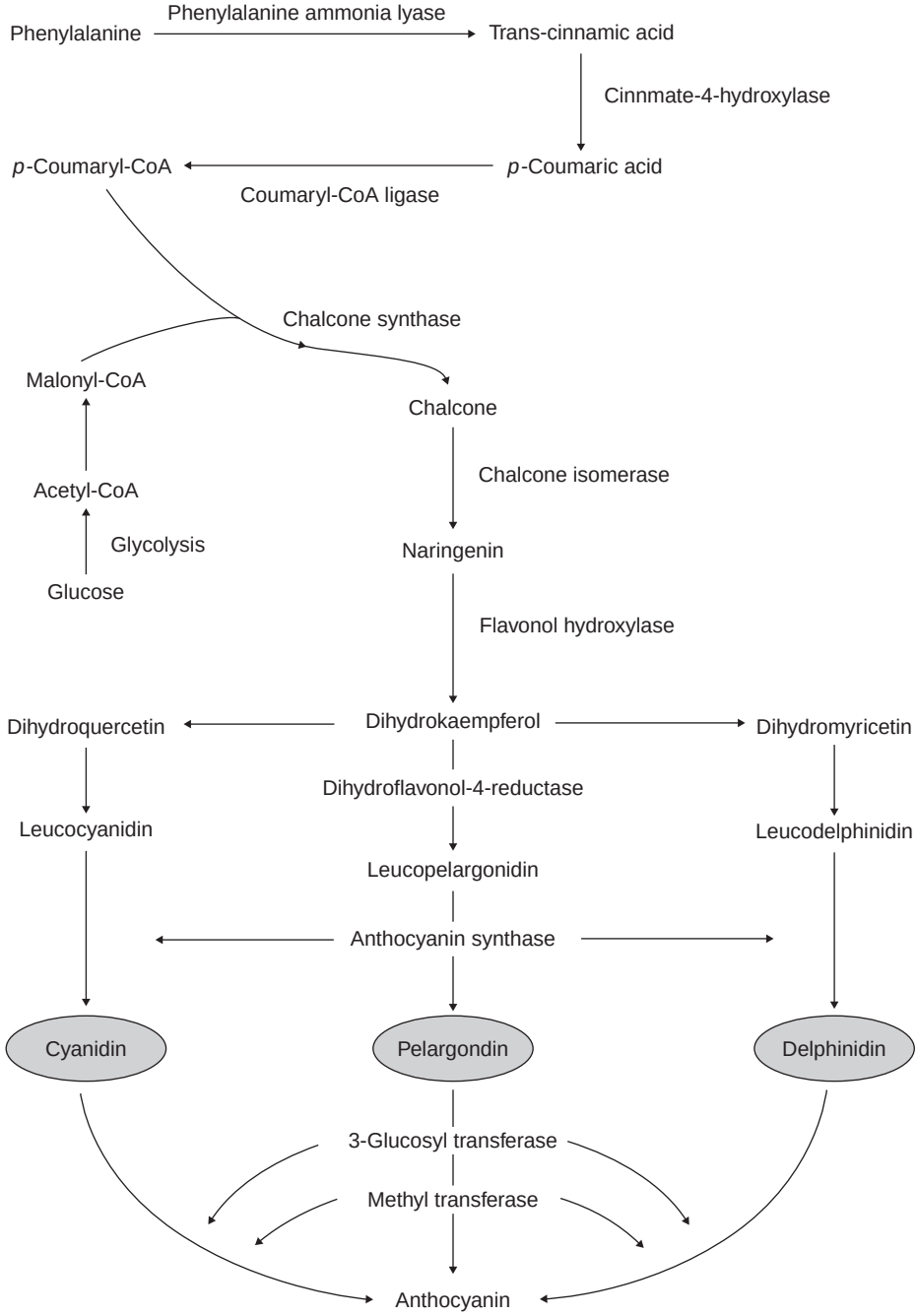
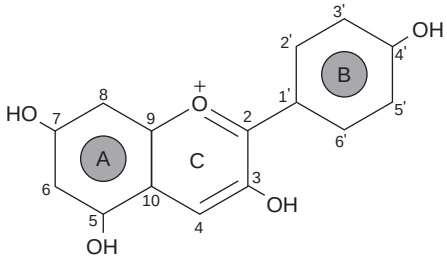
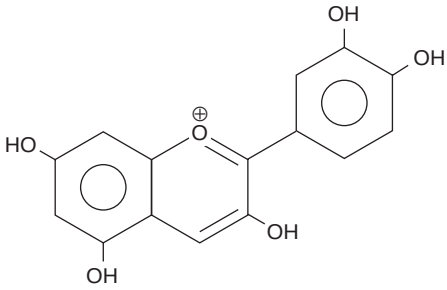


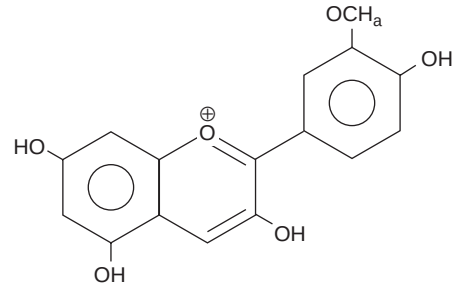
Fig. 3.8 Anthocyanin biosynthetic pathway in plants.



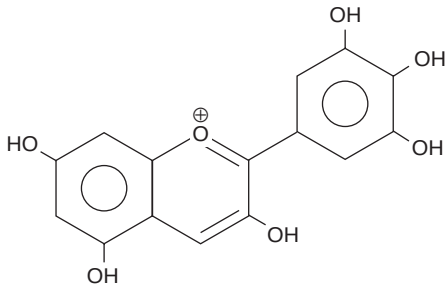
Pelargonidin



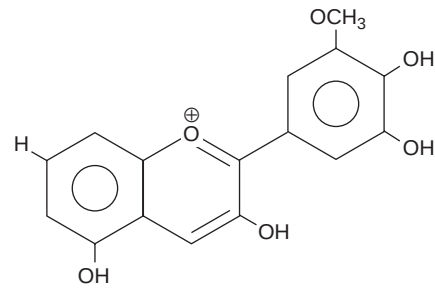
Cyanidin



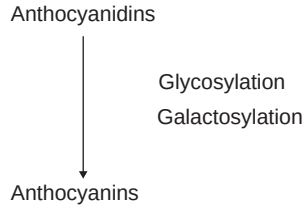
Peonidin

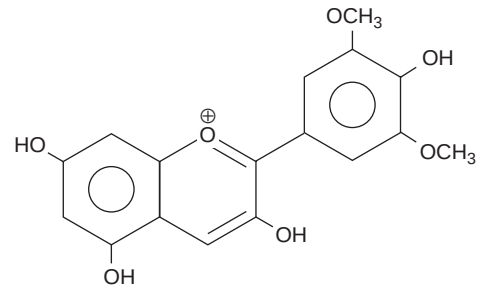


Delphinidin



Petunidin





Malvidin

Fig. 3.9 Some common anthocyanidins found in fruits and flowers.

coloration, and delphinidins provide the purple, mauve, and blue color characteristic to several fruits. The color characteristics of fruits may result from a combination of several forms of anthocyanins existing together, as well as the conditions of pH and ions present in the vacuole.

Anthocyanin pigments cause the diverse coloration of grape cultivars resulting in skin colors varying from translucent, red, and black. All the forms of anthocyanins, along with those with modifications of the hydroxyl groups, are routinely present in the red and dark varieties of grapes. A glucose moiety is attached at the 3 and 5 positions or at both in most grape anthocyanins. The glycosylation pattern can vary between the European (*Vitis vinifera*) and North American (*Vitis labrusca*) grape varieties. Anthocyanin accumulation occurs toward the end of ripening, and is highly influenced by sugar levels, light, temperature, ethylene, and increased metabolite translocation from leaves to fruits. All these factors positively influence the anthocyanin levels. Most of the anthocyanin accumulation may be limited to epidermal cell layers and a few of the subepidermal cells. In certain high-anthocyanin-containing varieties, even the interior cells of the fruit may possess high levels of anthocyanins. In the red wine varieties such as merlot, pinot noir, and cabernet sauvignon, anthocyanin content may vary between 1,500 and 3,000 mg/kg fresh weight. In some high-anthocyanin-containing varieties such as Vincent, Lomanto, and Colobel, the anthocyanin levels can exceed 9,000 mg/kg fresh weight. Anthocyanins are very strong antioxidants and are known to provide protection from the development of cardiovascular diseases and cancer.

Many fruits have a tart taste during early stage of development, which is termed as astringency, and is characteristic to fruits such as banana, kiwi, and grape. The astringency is due to the presence of tannins and several other phenolic components in fruits. Tannins are polymers of flavonoids such as catechin and epicatechin, phenolic acids (caffeoyl tartaric acid, coumaroyl tartaric acid, etc.). The contents of tannins decrease during ripening, making the fruit palatable.

3.4.3 Ester volatile biosynthesis

The sweet aroma characteristic to several ripe fruits are due to the evolution of several types of volatile components that include monoterpenes, esters, organic acids, aldehydes, ketones, alkanes, etc. Some of these ingredients specifically provide the aroma characteristic to fruits and are referred to as character impact compounds. For instance, the banana flavor is predominantly from isoamyl acetate, apple flavor from ethyl-2-methyl butyrate, and the flavor of lime is primarily due to the monoterpene limonene. As the name implies, ester volatiles are formed from an alcohol and an organic acid through the formation of an ester linkage. The alcohols and acids are, in general, products of lipid catabolism. Several volatiles are esterified with ethanol giving rise to ethyl derivatives of aliphatic acids (ethyl acetate, ethyl butyrate, etc.).

The ester volatiles are formed by the activity of the enzyme acyl CoA: alcohol acyltransferase or generally called as alcoholacyltransferase. In apple fruits, the major aroma components are ester volatiles (Paliyath et al., 1997). The alcohol can vary from ethanol, propanol, butanol, pentanol, hexanol, etc. The organic acid moiety containing the CoA group can vary in chain length from C2 (acetyl) to C12 (dodecanoyl). Alcoholacyltransferase activity has been identified in several fruits that include banana, strawberry, melon,

apple, etc. In banana, esters are the predominant volatiles enriched with esters such as acetates and butyrates. The flavor may result from the combined perception of amyl esters and butyl esters. Volatile production increases during ripening. The components for volatile biosynthesis may arise from amino acids and fatty acids. In melons, the volatile components comprise esters, aldehydes, alcohols, terpenes, and lactones. Hexyl acetate, isoamyl acetate, and octyl acetate are the major aliphatic esters. Benzyl acetate, phenyl propyl acetate, and phenyl ethyl acetate are also observed. The aldehydes, alcohols, terpenes, and lactones are minor components in melons. In mango fruits, the characteristic aroma of each variety is based on the composition of volatiles. The variety "Baladi" is characterized by the presence of high levels of limonene, other monoterpenes and sesquiterpenes, and ethyl esters of even numbered fatty acids. By contrast, the variety "Alphonso" is characterized by high levels of C6 aldehydes and alcohols (hexanal, hexanol) that may indicate a high level of fatty acid peroxidation in ripe fruits. C6 aldehydes are major flavor components of tomato fruits as well. In genetically transformed tomatoes (antisense phospholipase D), the evolution of pentanal and hexenal/hexanal was much higher after blending, suggesting the preservation of fatty acids in ripe fruits. Preserving the integrity of the membrane during ripening could help preserve the fatty acids that contribute to the flavor profile of the fruits, and this feature may provide a better flavor profile for fruits.

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Chapter 4

Biochemistry of Flower Senescence

Ajay Arora

4.1 Introduction

It is often opined that death and taxes are the only two inescapable aspects of the human existence, but Ernest Hemingway correctly noted that "the sun also rises." Plant senescence is the final event in the growth and development of a plant and ultimately leads to the death of a particular organ or whole plant. The senescence in plants is highly regulated, genetically programmed, and developmentally controlled process. This phenomenon involves structural, biochemical, and molecular changes that in many cases bear the hallmarks of programmed cell death. Plant hormones and environmental factors play an important regulatory role in senescence. Flower senescence has been described as the last stage of floral development, although in the life cycle of most plant species, it is not a final event, rather an integral process that allows the removal of a metabolically costly tissue (i.e., petal), after it has attracted pollinators for sexual reproduction, and signals the initiation of ovule development and seed production. At the end of their life, petals may wilt, lose color or abscise, or in some cases, remain on the flower stem, encasing and protecting the developing ovary. It is an actively ordered process that involves the synthesis of new RNAs and proteins and results in highly coordinated changes in metabolism and the programmed disassembly of cells.

All cut flowers are destined to die, and the challenge for postharvest researchers is to slow the processes controlling flower death to enable cut flowers to reach distant markets with a display life. Postharvest performance of cut flowers is affected by the developmental stage of a flower at harvest, prosenescence signals that originate from specific tissues within the flower (e.g., pollination-induced petal senescence), and stress-related metabolism (in response to temperature, wounding, nutrient starvation). Cut flower stems are removed from a source of nutrients, undergo water restrictions, and may be held at undesirable temperatures in the dark for days prior to sale. Plant hormones, membrane stability, water availability, cellular proteolysis, and carbohydrate metabolism act in concert to determine the differential rate of senescence for each floral organ. Currently, flowers can be grouped into several categories based on postharvest technologies that can extend their vase life (e.g., sensitivity to ethylene, chilling sensitivity, leafy stems, multiple/single flowers per stem, and woody stems).

Flower petals are ideal tissues for cell death studies as they are short lived, the tissue is relatively homogenous, chemical manipulation can be applied without substantial wounding (i.e., feeding through the vascular tissue), and the process of flower senescence has been shown to be a genetically programmed event (Xu and Hanson, 2000; Eason et al., 2002;

Hoeberichts et al., 2005). A great deal of recent research in this area has led to review and reevaluation of senescence and cell death in plant tissues (Rubinstein, 2000; Thomas et al., 2003; van Doorn and Woltering, 2004). To date, most genetic analyses of floral senescence have focused on changes that occur in mature flowers just prior to wilting or color change. However, senescence of one floral organ (e.g., petal) is part of a developmental continuum in the flower, preceded by tissue differentiation, growth and maturation of the petal, followed by growth and development of seeds, and co-coordinated by plant hormones. Cell death processes are thought to be regulated by anti- and pro-death proteins, which may be expressed throughout the life of the flower, providing for the most part a highly regulated homeostatic balance. Future genetic analyses of floral senescence are likely to identify the proteins that function to maintain a nonsenescent "youthful" state, and the "prosenescence" proteins which function to progress cell death. The past decade has seen increasingly rapid isolation and identification of senescence-associated genes from cut flower crops, with a somewhat slower movement toward understanding the function and significance of the gene products. Genome-wide searches for regulatory flower senescence genes have now been made in a number of flower species, for example, *Alstroemeria* (Breeze et al., 2004), carnation (Verlinden et al., 2002), chrysanthemum (Narumi et al., 2005), daffodil (Hunter et al., 2002), daylily (Panavas et al., 1999), rose (Channeliere et al., 2002), *Iris* (van Doorn et al., 2003), *Sandersonia* (Eason et al., 2002), and petunia (Jones et al., 2005). Characterizing generic patterns of gene expression has identified common processes that are linked with the progression of flower senescence (e.g., ethylene signaling and proteolysis). This approach will also be useful in identifying the order of molecular changes associated with flower senescence, thereby enabling researchers to accurately study cause and effect. This chapter focuses on molecular and genetic research published within the last one decade that has increased our understanding of the processes involved in or regulating flower senescence (e.g., ethylene, water quality, cytokinin, sugar, proteolysis, membranes, and cell walls), and its significance to the postharvest industry.

4.2 Petal senescence

In petals of cut flowers undergoing senescence, protein content falls, protease activity increases, lipid fluidity in the membranes declines, and respiration rate increases (van Doorn and Stead, 1997). Senescing carnation flowers exhibit a climacteric-like rise in ethylene production, and exposure of carnation flowers to exogenous ethylene induces in-rolling of petals, triggering ethylene synthesis, and inducing chemical and physical changes in microsomal membrane lipids of senescing petals (Bartoli et al., 1996). In chrysanthemum, which is nonclimacteric, ethylene does not play a role in flower senescence, with only minor changes in protein content and the proportion of major polypeptides, explaining the long postharvest life of chrysanthemum. Conditions inhibiting the action of that is by the supply of silver salt, sodium benzoate or boric acid, or the synthesis of ethylene, that is by the supply of aminooxyacetic acid (AOA), prolong the vase life of carnations (Serrano et al., 2001); an invertase inhibitor, apparently synthesized in wilting petals of a number of flowers (*Ipomoea*, alstroemeria, carnation, dahlia, gladiolus, petunia, and rose), affects the senescence of petals by blocking sucrose hydrolysis to glucose and fructose in the senescing tissue, which may control the translocation of sucrose from wilted petals to other organs of the flower. Petal abscission in rose petals is not affected by the water status unless the

plants reach a low water potential early on during vase life, neither inhibited by low light intensity nor affected by the Pr/Pfr ratio (van Doorn and Vojinovic, 1996).

4.3 Ethylene and flower senescence

Traditionally, flowers (like fruits) are categorized as being climacteric or nonclimacteric. In climacteric or ethylene-sensitive flowers such as carnations, *Petunia*, *Gypsophila*, and orchids, senescence is accompanied by a sudden, transient increase in ethylene production and respiration, while treatment of nonsenescent flowers with ethylene rapidly induces petal senescence. In nonclimacteric flowers such as gladiolus, tulip, and iris, generally, no increases in ethylene production and respiration are apparent during flower senescence, and exogenous ethylene has little or no effect on petal senescence. In these latter species, ethylene may, however, have severe effects on other plant parts such as bulbs or corms (Kamerbeek and De Munk, 1976). Knowledge about ethylene sensitivity of flower species is necessary to predict the effects of, for example, mixed storage and transport of flowers with fruit species, to predict the usefulness of antiethylene treatments and to direct breeding programs toward a better flower vase life. With respect to petal senescence, sensitivity to ethylene was found to be roughly determined at the plant family level. High sensitivity is found in, for example, Campanulaceae, Caryophyllaceae, Geraniaceae, Labiatae, Malvaceae, Orchidaceae, Primulaceae, Ranunculaceae, and Rosaceae species; low sensitivity is found in Compositae and Iridacae species and in most of the Amaryllidaceae and Liliaceae species. Sensitivity of species within one plant family is generally comparable (Woltering and van Doorn, 1988). As a rule of thumb, this classification is satisfactory, although there are notable exceptions.

In flowers where petal senescence is independent of ethylene (e.g., in many ephemeral flowers, in short-lived flowers from, e.g., Iridaceae and Liliaceae plant families, and in long-lived flowers of Compositae family), other aspects of flower development such as flower opening, ovary development, or pedicel elongation may be responsive to ethylene. As an example, petal senescence in iris flowers is virtually insensitive to ethylene; however, treatment with very low concentrations of ethylene may seriously inhibit flower opening as a result of ethylene-induced growth inhibition of the flower pedicel. Depending on the species, ethylene, either applied or endogenously produced, may induce various processes (Woltering and van Doorn, 1988). In many flowers, ethylene hastens senescence of petals that initially stay attached to the flower. This type of senescence is found in, for example, Orchidaceae (*Cymbidium*, *Dendrobium*), Campanulaceae (*Campanula*, *Trachelium*), and Caryophyllaceae (*Dianthus*, *Gypsophila*). In other flowers, ethylene may induce abscission of fully turgescent, nonsenescent petals or of whole corollas. This type of effect is found in, for example, Geraniaceae (*Geranium*), Ranunculaceae (*Aconitum*, *Delphinium*), Rosaceae (*Rosa*, *Potentilla*), and Scrophulariaceae (*Antirrhinum*, *Veronica*). In contrast to petal senescence, which is also found in ethylene insensitive species, ethylene insensitive abscission of petals seems extremely rare in plant kingdom (Sexton et al., 2000).

Apart from these effects of ethylene on petal senescence and petal abscission, ethylene may also stimulate abscission of whole flowers and flower buds as in *Hibiscus, Begonia, Clerodendron, Fuchsia*, and *Agapanthus*, or may stimulate abscission of whole inflorescences (*Beloperone*, *Pachystachus*) (Woltering, 1987). Strikingly, in flowers with a relatively long life, ethylene has evolved as a trigger of senescence or abscission. In such

flowers, pollination often triggers an increase in ethylene production and subsequent rapid senescence and it has been suggested that ethylene may have evolved as a mechanism to terminate flower life after successful pollination as a way to benefit survival of the species (van Doorn, 2001). In ephemeral and short-lived flower species, such a mechanism apparently is not beneficial as the life of individual flowers is very short. Similarly, in Compositae species, with numerous flowers in one flower head, continuous visits of pollinators are required to fertilize all individual flowers and the senescence of pollinated flowers would not be beneficial.

Although ethylene sensitivity is roughly fixed at the plant family level, still marked differences may exist between species and cultivars within one family. Several carnation cultivars (e.g., Chinera, Epomeo, and Ginevra) derived from crosses involving a long-life noncommercial breeding line have been described with reduced ethylene sensitivity compared to the cultivar White Sim. The vase life of these cultivars was negatively correlated with their ethylene sensitivity (van Doorn et al., 1993). Basic properties of ethylene receptors (number and affinity) were thought to be similar in cultivars Chinera and White Sim (Woltering et al., 1993). The reduced response of cultivar Chinera to ethylene was thought to be regulated at a point beyond the receptor. In other carnation cultivars (Sandra, Sandrosa), the prolonged vase life was related to a decreased activity of the ethylene biosynthetic pathway and not to reduced ethylene sensitivity (Mayak and Tirosh, 1993). Also, other carnation cultivars with either a long vase life or decreased ethylene sensitivity have also been described (Brandt and Woodson, 1992). Recently, another cultivar with a long vase life (White Candle) was described (Nukui et al., 2004). This cultivar showed decreased expression of ACC-synthase genes and repressed ethylene production in the gynoecium but showed a normal response to exogenous ethylene. In another species within the Caryophyllaceae, *Dianthus barbatus*, different genetic lines with greatly reduced ethylene sensitivity were described (Friedman et al., 2001). Also in roses, marked differences in ethylene sensitivity exist between varieties. In some cultivars, petals show marked growth variations in response to ethylene (Reid et al., 1989), whereas in other cultivars this effect is absent. In addition, in miniature potted roses, a range of ethylene sensitivities was found from almost insensitive to highly sensitive (Muller et al., 1998). The above examples clearly underline that the distinction between ethylene sensitive and insensitive senescence needs to be handled with care. On the one hand, ethylene-insensitive flowers may show effects of ethylene in processes other than petal senescence or abscission, while on the other hand, considerable cultivar variation may exist so that cultivars of a given species may range from insensitive to very sensitive. Controlling ethylene effects through interference with ethylene perception may therefore be very beneficial in a variety of cases, both in ethylene-sensitive and ethylene-insensitive flowers.

As said earlier, ethylene plays a crucial role in the senescence of "ethylene-sensitive" flowers, coordinating senescence pathways and regulating floral abscission. Compatible pollination triggers a series of postpollination events, including ovary growth, floral collapse (wilting, abscission), and petal color change that are regulated by tissue-specific production and sensitivity of ethylene (O'Neill, 1997). Antiethylene treatments are common place in postharvest chains owing to the well-known damage and decay that free ethylene promotes in horticultural produce. The biosynthetic pathway for ethylene has been fully elucidated in higher plants, and plants with mutations that affect the perception or signal transduction of ethylene (namely, *Arabidopsis* and tomato) have been used to define the ethylene signaling

cascade (Arora, 2005; Chen et al., 2005). Genes that control ethylene production, ethylene sensitivity and genes that are affected by the presence of ethylene have been identified in cut flowers (Kosugi et al., 2000; Muller et al., 2002; Verlinden et al., 2002; Shibuya et al., 2004; Iordachescu and Verlinden, 2005). Chemical inhibitors have been used to study biosynthesis of ethylene and ethylene activity; AVG (aminoethoxyvinylglycine) and AOA (aminooxyacetic acid) inhibit ethylene biosynthesis; STS (silver thiosulfate), NBD (2,5-norbornadiene), and 1-MCP (1-methyl cyclopropene) bind and block the ethylene receptor. Currently, the vase life of carnation flowers can be extended by treating cut stems with inhibitors of ethylene biosynthesis (e.g., AOA), ethylene response or receptor inhibitors (e.g., STS, 1-MCP), and high temperature (Verlinden and Woodson, 1998) (a treatment normally exclusive to disinfestation procedures has also been shown to improve the vase life of carnations by reducing ethylene activity). Although these chemical treatments are effective at delaying postharvest senescence, comparative analyses of the senescence of transgenic and wild-type carnations showed that genetic modification for ethylene insensitivity was more effective than chemical treatment for vase life extension (Bovy et al., 1999). In recent years, petunia has proved to be an ideal model system for studying the regulation of postharvest flower senescence. The plants have a relatively short regeneration cycle and can be grown year-round and manipulated by pruning to produce multiple floral buds per plant for postharvest experiments. Petunia flower senescence is sensitive to ethylene and induced following pollination, although emasculated flowers are often used for postharvest experiments (Jones et al., 2005). The development and senescence of individual flowers have been fully characterized for this purpose (Xu and Hanson, 2000). It has been possible to study the ethylene-insensitive regulatory pathways in petunia flowers using plants that are genetically modified for insensitivity to ethylene (Wilkinson et al., 1997). Comparative analysis of flower senescence in ethylene-insensitive and wild-type petunia plants has shown that ethylene differentially regulates individual cysteine protease genes during flower senescence, supporting the hypothesis that senescence-induced gene expression in petals occurs via ethylene-dependent and -independent signaling pathways (Jones et al., 2005). Horticultural performance of transgenic ethylene-insensitive petunias has provided valuable information about other developmental programs that ethylene regulates, highlighting those that may hinder the exploitation of ethylene-insensitive cut flower crops in the future, for exmaple, poor rooting (Clark et al., 1999) and lower disease resistance (Shaw et al., 2002). Ethylene is perceived by plants when it binds a membrane-localized protein known as a receptor and is activated upon binding of ethylene and transmits the ethylene signal through a series of steps (Stepanova and Alonso, 2005). Subsequent activation of a transcription factors leads to induction of ethylene-responsive genes. Knowledge of ethylene receptors has advanced our understanding of how ethylene inhibitors (such as cyclopropenes) function. Cyclopropenes (gaseous ethylene receptor inhibitors) inactivate ethylene receptors by binding to, and excluding ethylene from the binding site. When the ethylene receptors of cut flowers are blocked with 1-MCP, 1-hexylcyclopropene or 1-octylcyclopropene, the flowers have extended vase lives, as they cannot perceive ethylene (Kebenei et al., 2003). The effectiveness of these compounds in delaying the onset of senescence in cut flowers is related to the number and turnover rate of ethylene receptors and the concentration and stability of the gaseous inhibitors. 1-MCP (*SmartFresh*, AgroFresh Inc., PA) is now commercially available as a replacement for silver thiosulfate in the floriculture industry, and sustained-release systems are being developed (Macnish et al., 2004).

Gladiolus is an ethylene-insensitive flower. Exogenous ethylene and ethylene inhibitors have no effect on the petal senescence process. To study which processes in gladiolus are associated with changes in ethylene perception, two types of gladiolus genes, named *GgERS1a* and *GgERS1b*, respectively, homologous to the *Arabidopsis* ethylene receptor gene ERS1 were isolated by Arora et al. (2006). GgERS1a is conserved in terms of exon numbers and intron positions, whereas GgERS1b is almost same with GgERS1a except lacking a 636-nucleotide frame encoding the first and second histidine kinase (HisKA) motifs. The sequence data on full length genomic DNA indicated that both GgERS1a and GgERS1b were spliced from different genomic DNA. As the result of mRNA expression study, in spite of lacking the two significant motifs, the expression of GgERS1b dramatically changed with advance in petal senescence, whereas the level of GgERS1a was expressed highly and constitutively. The result suggests that both the genes possess a significant role for the subfunctionalization process to provide ethylene insensitivity in gladiolus flowers (Arora et al., 2006).

Nitric oxide (NO) gas is also effective in extending the postharvest life of flowers through modulating endogenous ethylene activity (Badiyan et al., 2004). The gaseous nature of NO, however, requires postharvest infrastructures that may not always be readily available, and therefore the treatment will be less suitable than liquid-pulsing solutions. To this end, an NO donor compound (DETA/NO) applied in the vase water has been used to extend the vase life of flowers. The effectiveness of DETA/NO across ethylene-sensitive and -insensitive flowers suggests that it may have significant commercial application in the future (Badiyan et al., 2004). The mode of action of NO in delaying the onset of flower senescence has yet to be studied at the molecular level.

An experiment was conducted to study the effect of 5-sulfosalicylic acid (5-SSA) on the vase life of cut flowers of gladiolus (Ezhilmathi et al., 2007). The vase solution having 5-SSA significantly increased cumulative uptake of vase solution, vase life, number of opened florets, and decreased the number of unopened florets compared to the controls. Spikes kept in vase solution containing 5-SSA also exhibited lower respiration rates, lipid peroxidation, and lipoxygenase activity, and higher membrane stability, soluble protein concentration, and activity of superoxide dismutase and catalase. Results suggest that 5-SSA increases vase life by increasing the reactive oxygen species-scavenging activity of the gladiolus cut flowers.

4.4 Ethylene action and methods for inhibiting ethylene responses

A range of methods are available for preventing the deteriorative effect of ethylene on postharvest characteristics of ornamental crops. Interfering with the plant's response to ethylene can in principle be achieved by (1) inhibition of the plant's own ethylene production; (2) blocking the binding of ethylene to its receptor; and (3) by blocking the plant's reaction to the binding of ethylene to the receptor.

Interference with the biosynthesis of ethylene in ornamental plants can be achieved by blocking components of the ethylene synthesis pathway. During many years, several chemicals have successfully been used by the floral industry. AVG (1-aminoethoxyvinylglicine) and AOA (aminooxyacetic acid), both inhibitors of the conversion of *S*-adenosyl-methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) have been shown effective in blocking the increase in ethylene production that accompanies senescence in a variety of

ethylene-sensitive flowers, and a number of commercial products based on these chemicals for treatment of carnation and orchid flowers have entered the market (Son et al., 1994; Woltering and Harkema, 1994). However, blocking ethylene effects at the receptor level is more effective as it will protect against both endogenous and exogenous ethylenes (Serek and Reid, 1993). Several ethylene antagonists have been discovered during the last 30 years, and some of them have successfully been used by the floral industry to block ethylene responses. Another approach to interfere with ethylene is the use of molecular breeding techniques, in which ethylene responses are modified in transgenic plants. Central in the synthesis of ethylene in plants are two enzymes: 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO), formerly known as ethylene-forming enzyme (EFE) (Kende, 1993). The production of ethylene is autocatalytic in many plants, meaning that the presence of ethylene in the atmosphere or in the plant tissue causes a positive feedback, leading to a rise in the production of the hormone. The key protein responsible for the reactions to ethylene is the ethylene receptor, which consists of a family of membrane-embedded proteins that bind ethylene. In *Arabidopsis*, five members of this family have been identified: ETR1, ETR2, ERS1, ERS2, and EIN4. The first receptor to be identified was ETR1 (Chang et al., 1993), and homologs to this and some of the other proteins have been found in a number of other plants. Later work by Chang and others have elucidated the function of this receptor, as well as other proteins involved in the ethylene response pathway. These results can be summarized as follows: the ethylene signaling pathway is initiated by a family of membrane-embedded proteins (receptors) leading via a signal transduction cascade to altered gene expression patterns. The ethylene receptors are thought to exist as dimers that belong to the so-called two component histidine kinase receptor family. The receptors are negative regulators of ethylene responses, which mean that ethylene binding represses receptor signaling. When ethylene is not present, the receptor activates a protein called CTR1. CTR1 is a serine/threonine kinase and is presumably part of a mitogen-activated protein kinase signaling cascade, a highly conserved signaling route known to regulate a variety of cellular processes in different organisms. In the absence of ethylene, CTR1 is active and represses further signaling; when ethylene is present and binds to the receptor, this binding presumably induces a conformational change resulting in inactivation of CTR1, thereby releasing the repression on the signal transduction chain leading to the activation of transcription factors and the genes responsible for ethylene effects (Chang, 2003). For any reason the receptor is not capable of binding ethylene, CTR1 will be active and the signaling cascade will be repressed. ETR1 was first identified in *Arabidopsis* by its dominant mutant, called etr1-1 that does not show the normal triple response of seedlings to ethylene (Chang et al., 1993). It has been shown that this mutant protein does not bind ethylene (Hall et al., 1999). The dominant nature of this mutant can be understood considering that the different receptors feed into the same signaling cascade. The presence of one type of actively signaling receptor and, hence, activated CTR1, apparently is sufficient to repress the signaling cascade leading to gene expression and subsequent ethylene symptoms even when other receptor types are inactivated. In addition, increased dosage of wild-type alleles in triploid lines led to the partial recovery of ethylene sensitivity, indicating that dominant ethylene insensitivity may involve either interactions between wild-type and mutant receptors or competition between mutant and wild-type receptors for downstream effectors (Hall et al., 1999). Through this knowledge, we can understand that the plant's reaction to ethylene can be prevented by (1) application of a chemical that blocks the receptor in such a

way that ethylene cannot bind, but without inducing the conformational change as ethylene would or (2) breeding plants that express a mutant receptor that, like etr1-1, does not bind ethylene. Both of these means would cause at least part of the receptors to stay "active" in the presence of ethylene, thereby blocking the signal transduction events leading to gene expression. Unless the action in some way can be restricted to specific tissues, the result of either method would be that all reactions to ethylene will be blocked. As discussed below, this can have some unwanted effects.

4.4.1 Blocking ethylene perception by chemicals

Increased carbon dioxide and decreased oxygen concentrations are well-known antagonists of ethylene action, and various packaging and storage methods (modified atmosphere, controlled atmosphere) for fresh horticultural products partly rely on this principle. The biochemical background of these effects, however, is still largely unknown. Ethylene physiologists used CO_2 for years as an inhibitor of ethylene action in testing the ethylene involvement in processes, which they examined and, till today, CO_2 is widely regarded as a compound that interferes with ethylene sensitivity. The original hypothesis of Burg and Burg (1967) that CO_2 and ethylene may compete for the same binding site, however, has found not to be true. In ethylene binding experiments, it was shown that CO_2 could not displace ethylene from the receptor, and by using CO_2 in combination with 1-MCP (a proved inhibitor of ethylene perception as discussed below), it was clearly shown that CO_2 mainly acts independent of the ethylene receptor (de Wild et al., 2005). The most likely site of action of CO_2 in inhibiting ethylene effects is at the activity of ACC synthase. An increased ACC-synthase activity and, hence, an increased ethylene production is often a major effect of ethylene treatment; the lack of increased ethylene production under elevated CO_2 levels may seem to suggest that ethylene perception is altered. A similar reasoning may hold true for decreased O_2 levels. Despite the general idea that oxygen is required for ethylene perception, the observed decreased responses to ethylene under low-oxygen conditions may be related to the effect of low oxygen on processes other than the binding of ethylene to the receptor. For instance, low oxygen is known to suppress ACO activity, thereby blocking the autocatalytic ethylene production. Although the effect of ethylene certainly is less pronounced under elevated CO_2 or decreased O_2 levels, this effect may be on processes beyond the actual perception of ethylene.

Lowering the temperature also dramatically reduces ethylene effects. For carnation flowers it was calculated that for each 10 degrees drop in temperature, ethylene response decreases over 10 times ($Q_{10} = 11.3$), due to two different processes. At lower temperatures, the time required for a given response (irreversible wilting) increased with a Q_{10} of 2.7. In addition to this, affinity of binding sites to ethylene was shown to decrease with a Q_{10} of 4.2 (Woltering et al., 1994). This shows that at low temperatures, not only the response to ethylene, but also the perception of ethylene at the receptor level are suppressed. Together with a decreased ethylene production at lower temperatures ($Q_{10} = 2.7$), this explains the dramatic effect of temperature on ethylene effects and suggests that once stored at low temperature, other ways to block ethylene effects may not be necessary. Although controlled atmosphere storage is a common practice for many types of fruit, it is currently not a common practice for storage of flowers or potted plants. Reid and Serek (1999) recommend for carnations and roses an elevation of CO_2 to the level of 3% for inhibition

of ethylene action. The elevation of CO_2 level was accompanied by a reduction of O_2 to 2% and storage at low temperature (0°C). The authors reported that the potential benefit of this treatment as well as a commercial use is very limited.

4.4.2 Chemical inhibition of ethylene perception

4.4.2.1 2,5-Norbornadiene

In the early seventies, several alkenes have been reported by Sisler and Pian (1973) as effective ethylene antagonists. The most stabile of these compounds was 2,5-norbornadiene (2,5-bicyclohepta-2,5-diene; NBD). NBD is a liquid with a low boiling point (89°C) that easily vaporizes at room temperature, which makes it easy to treat plant material in airtight chambers. NBDs effectiveness in preventing ethylene effects was tested on different plant materials, including carnation flowers (Sisler et al., 1986). Continuous treatment of carnation flowers with NBD, even at lowest concentration tested (500 μL/L), strongly increased vase life of the flowers and delayed the onset of climacteric ethylene production. Higher concentrations of NBD inhibited ethylene binding in carnation flowers and leaves with 66 and 86%, respectively. Even though NBD is commercially available, its very disagreeable odor limits its use to scientific investigations (Sisler and Serek, 1999). Additionally, NBD is blocking the receptor in a competitive manner and, therefore, has to be continuously present to counteract ethylene effects. Therefore, NBD has very limited potential for commercial use (Sisler et al., 1990).

4.4.2.2 Silver thiosulfate

The silver ion has proved to be a potent inhibitor of ethylene action in ornamentals. Ethylene-binding studies using radio-labeled ethylene have shown that STS treatment blocks the binding of ethylene (Sisler et al., 1986). ETR1 has been shown to contain a copper ion that coordinates the binding of ethylene (Rodrigez et al., 1999), and the action of STS may be related to the exchange between copper and silver ions in the receptor protein. The silver-containing receptor apparently is locked in such a state that ethylene cannot bind and induce a conformational change (Knee, 1995). For a number of years, STS was the only compound used commercially to protect ornamental crops against ethylene at the receptor level. STS is generally applied as a pretreatment solution to cut flowers. The persistence and mobility of STS allows very short pulse treatments. In potted plants STS is applied as an aqueous spray. Beneficial effects of STS are reported for a great variety of cut flowers and potted plants. STS treatment prevents petal senescence induced by ethylene and prolongs the vase life in, for example, carnation, *D. barbatus*, *Gypsophila, Matthiola, Trachelium, Physostegia*, and several orchid species. However, byproducts of use can lead to environmental contamination.

4.4.2.3 Diazocyclopentadiene

Years after the discovery of NBD and STS, new compounds have been tested for extending the postharvest life of ornamental crops. Diazocyclopentadiene (DACP) has been tested on different ornamental crops, like carnations, miniature roses, and sweet pea flowers (Serek et al., 1994). DACP binds weakly to the ethylene receptor (Sisler and Serek, 1997), but under fluorescent light, DACP decomposes to unknown compounds and becomes a very effective ethylene receptor blocker. The active product of decomposition of DACP has not

yet been identified. The study of Sisler et al. (1993) reported on comparison of the effects of STS and preirradiated DACP on preventing ethylene effects in cut carnation flowers (*Dianthus caryophyllus* "White Sim"). The differences between treatments did not appear during the 12 days of observation. Additionally, DACP delayed and reduced endogenous ethylene production. In the study with sweet pea flowers, Sexton et al. (1995) investigated the effect of DACP on bud drop from cut sweet pea flower spikes (*Lathyrus odoratus* L). DACP delayed both wilting and abscission of buds; however, it did not stop the processes as effectively as it was reported for carnation petal senescence (Sisler et al., 1993). The ethylene binding constants (*Kd* values) in rose petals and leaves obtained indicates noncompetitive inhibition by irradiated DACP, probably because DACP covalently attached to the binding site when the diazo groups decomposed (Serek et al., 1994). This volatile compound has been documented as a very strong ethylene action inhibitor; however, its instability and explosive character makes it an unlikely candidate for commercial use.

4.4.2.4 1-Methylcyclopropene

The spectacular effect of 1-methylcyclopropene (1-MCP) has been well documented in a range of ornamental species. The commercial producers of ornamental products in many countries are already benefiting from this new development. 1-MCP is a cyclic olefin, which seemingly irreversibly binds to ethylene receptors and prevents ethylene from inducing a conformational change. The compound is nontoxic, odorless, stable at room temperature, and has been shown to protect many cut flowers, potted plants, and other horticultural commodities against ethylene. The physical and chemical properties, as well as synthesis and mode of action of 1-MCP, have been reviewed by Sisler and Serek (2003). 1-MCP has been patented in 1996 (Sisler and Blankenship, 1996) and is commercially produced by AgroFresh Inc. (*SmartFresh*).

4.4.2.5 Other cyclopropenes

The effect of 1-MCP pretreatment generally has a limited duration. This most likely is due to breakdown of receptor protein and the synthesis of new receptor molecules that are, due to absence of the gas, not protected against ethylene (Sisler et al., 1996b). Further development and testing of new compounds to achieve a broader working spectrum has therefore been performed (Sisler et al., 2001). A number of 1-MCP-related compounds have been investigated in order to find the most effective ethylene antagonists. Cyclopropene (CP) and 3,3-dimethylcyclopropene (3,3-DMCP) have been tested in carnation and *Campanula* (Sisler et al., 1996a); 3-methylcyclopropene (3-MCP) has been tested in *Campanula* and *Kalanchoe* (Sisler et al., 1999). All these compounds were found to be efficient in protecting plant tissue from the effect of ethylene, though the necessary concentrations varied. None of these compounds performed better than 1-MCP. Contrary to these results, methylene cyclopropane has been found to induce most of the same effects as ethylene does (Sisler et al., 1996b). Apparently, this compound acts as an agonist instead of an antagonist. Later investigations showed that 1-substituted cyclopropenes (like 1-MCP) performed better than any other compounds, and a range of such compounds substituted with various carbon chains at the 1-position has been tested by Sisler et al. (2003). This has resulted in a number of compounds with very desirable qualities. In this study, compounds with all possible linear saturated side chains from methyl (CH_3) to decyl ($(CH_2)9CH_3$) were tested. The general result was that the longer the side chain, the better the performance.

1-Decylcyclopropene (1-DCP) showed the best performance: both in terms of a very low necessary concentration for maximum protection, 0.3 nL/L; and the longest duration of action, 36 days. Although these studies were made in banana fruits, there is little doubt that these compounds will be very effective in protecting flowers from ethylene effects as well. One could speculate that the longer lasting effect could be due to the hydrophobic side chain anchoring the compound to the cell membrane, thereby preventing the molecule from getting lost from the cell surface and, at the same time, allowing a larger amount of compound to be bound to the tissue. This slow-release effect could then result in the compound being bound to ethylene receptor molecules, synthesized long after the initial treatment, thereby prolonging the effect. In flowers the most thoroughly investigated of these compounds is 1-octylcyclopropene (1-OCP). It has been tested in *Kalanchoe blossfeldiana* (Sisler and Serek, 2003) and *L. odoratus* (Kebenei et al., 2003). Generally, this compound was equally or slightly more efficient than 1-MCP (compared on a concentration v/v basis) as a protective agent against ethylene action in flowers. Also, 1-hexylcyclopropene (1-HCP) has been shown to be effective in *Kalanchoe*, though in somewhat higher concentrations (Kebenei et al., 2003).

4.5 Regulation of senescence-related gene expression by ethylene

The plant hormone ethylene has been implicated in the regulation of both fruit ripening and leaf and flower senescence (Abeles et al., 1992). A number of senescence regulated (SR) and ripening-related genes have been found to be upregulated by the exogenous application of ethylene (Davies and Grierson, 1989; Lawton et al., 1990; Grbic and Bleecker, 1995; Weaver et al., 1998). Treatment of preclimacteric flowers with ethylene results in the induction of all the SR genes identified from carnation (Jones and Woodson, 1999). In tomato, the highest level of expression of pTOM genes in fruit was detected at the orange stage when ethylene production was highest and enhanced expression in leaves coincided with the first visible symptoms of leaf yellowing. Treatment with exogenous ethylene resulted in increased expression of pTOM genes in fruit and leaves, providing evidence that ethylene-controlled gene expression is involved in both fruit ripening and leaf senescence (Davies and Grierson, 1989).

Never ripe (NR) tomatoes, which are insensitive to ethylene due to a mutation in the ethylene receptor, produce fruit in which ripening is inhibited, have flower petals that do not senesce, and have leaves with delayed leaf yellowing (Lanahan et al., 1994). In the fruit of NR tomatoes, ripening-related transcripts accumulate too much lower levels than in wild-type fruit (DellaPenna et al., 1989). *Arabidopsis* plants with a mutated ethylene receptor, *etrl-1*, also show delayed leaf senescence but, once initiated, the process of senescence and the level of SAG expression is similar to that detected in wild-type leaves (Grbic and Bleecker, 1995). The treatment of tomato plants with the ethylene action inhibitor, silver thiosulfate, delays both fruit and leaf senescence and greatly reduces the expression of the mRNAs for *pTOM31*, *pTOM36*, and *pTOM137* and to a lesser degree *pTOM13*, *pTOM66*, and *pTOM75* in both fruit and leaves (Davies and Grierson, 1989). Treatment of carnation flowers with the ethylene action inhibitor, norbornadiene (NBD), delays the age-related accumulation of all SR genes except *SR5* (Lawton et al., 1990; Woodson et al., 1992, 1993). Treatment with NBD also reduces the basal levels of *DCCP1* transcript in petals

(Jones et al., 1995). These experiments indicate that while many SR genes are regulated by ethylene, they are also regulated by developmental or temporal cues.

The ability of plant organs to respond to exogenous ethylene appears to be developmentally regulated as the enhanced expression of SAGs in ethylene-treated leaves is greatest in old leaves and not detectable or only moderately induced in young green leaves (Weaver et al., 1998). Immature tomato fruits and flowers also do not respond to exogenous ethylene with ripening or petal senescence. This ethylene treatment does not induce the expression of ripening-related genes in immature green fruit or SR genes in petals from flowers in the bud stage (Lawton et al., 1990). While some flowers like daylily and nonclimacteric fruits like strawberry are not regulated by ethylene, it is clear that ethylene plays a regulatory role in both senescence and fruit ripening through the transcriptional regulation of SR genes.

The observed differences in the timing of the response of various SR genes to external stresses and plant hormones indicate that some of the SR genes may respond directly to stress, while others may be regulated by senescence that results from the stress or hormone application (Weaver et al., 1998). Further characterization of the response of SR genes to various stresses will help to identify those genes that are primarily responsive to senescence and are thus key regulators of senescence. There are many genes that are upregulated during senescence and involved in the activation and coordination of senescence; the downregulation of genes that act as repressors of senescence may play an equally important role in regulating senescence. Currently, most of the genes identified as downregulated during senescence are genes involved in photosynthesis (John et al., 1997). Transcript levels for the pea homolog of the *defender against apoptotic death* (*dad*) gene, a gene known to function as a repressor of programmed cell death (PCD) in *Caenorhabditis elegans* and mammals, have been found to decrease during flower development (Orzaez and Granell, 1997), while the *dad-1* cDNA from rice can rescue temperature-sensitive *dad-1* mutants of hamster from PCD. Yamada et al. (2004) isolated a homolog of the potential antiapoptotic gene, defender against apoptotic death (DAD1) from gladiolus petals as a full-length cDNA (*GlDAD1*), and investigated the relationship between its expression and the execution processes of PCD in senescing petals. RNA gel blotting showed that *GlDAD1* expression in petals was drastically reduced, considerably before the first visible senescence symptom (petal wilting). A few days after downregulation *GlDAD1* expression, DNA and nuclear fragmentation were observed, both specific for the execution phase of PCD, but the function of the dad gene in plant senescence is still not very clear.

4.6 Genes involved in ethylene biosynthesis and perception

The biosynthesis and perception of the plant hormone ethylene are known to modulate specific components of leaf senescence, fruit ripening, and flower senescence (Grbic and Bleecker, 1995). All three processes are also known to be accompanied by increases in the synthesis of ethylene (Abeles et al., 1992), and therefore it is reasonable to assume that SR genes would include those involved in ethylene biosynthesis. Two enzymes, 1-aminocyclopropane-l-carboxylate (ACC) synthase and ACC oxidase, have been identified as catalyzing rate-limiting steps in ethylene biosynthesis (Kende, 1993). While no ACC-synthase genes have specifically been isolated by differential screening of senescing or ripening tissues, three SR clones have been identified that encode ACC oxidase; these include *pTOM13* from tomato (Hamilton et al., 1991); *SR120* from carnation petals

(Wang and Woodson, 1991); and *pBANUU10* from banana (Medina-Suarez et al., 1997). While *pTOM13* is upregulated in leaves, flowers, and fruits, *SR120* is flower-specific (Barry et al., 1996). On the identification of additional ACC-synthase and ACC-oxidase genes, transcriptional upregulation has been reported during flower and leaf senescence and fruit ripening in many species (Abeles et al., 1992). While the ethylene biosynthetic pathway is well established, components involved in ethylene perception and signal transduction have only recently been identified. Initial studies on the expression of genes encoding the ethylene receptor report that specific receptor genes are upregulated during fruit ripening and senescence, while others appear to be constitutively expressed in multiple tissues (Tieman and Klee, 1999).

During the last 10 years, enormous progress has been made both in the understanding of the mode of action of ethylene at the molecular level as well as in the translation of this knowledge into strategies to counteract the detrimental effects of ethylene on ornamental products. Currently, 1-MCP is entering the market worldwide to treat a variety of ornamental products and, genetically engineered ornamentals, with either a decreased ethylene production or perception, and subsequent superior postharvest performance have been produced. Given the changing public and political opinions with regard to genetically modified organisms, it is expected that the latter products will also soon enter the flower market.

4.7 Role of plant growth regulators in flower senescence

4.7.1 Cytokinins

Flower senescence process, like whole plant senescence, is an active one that is executed via a defined genetic program. Once a flower has been pollinated or is no longer receptive to pollination, the programmed senescence of petals allows for the removal of a metabolically costly tissue whose sole role in sexual reproduction is to attract a pollinator. In many flowers, pollination-induced petal senescence results in the degradation of macromolecules from a structure that is no longer needed and allows for the remobilization of nutrients to developing tissues like the ovary (Stead, 1992).

Senescence is accompanied by changes in endogenous ethylene, abscisic acid (ABA), and cytokinins, and these changes are believed to mediate signaling events that control the process. In ethylene-insensitive plants like daylily, ABA is thought to be the primary hormonal regulator of flower senescence, and exogenous application of ABA accelerates visual senescence symptoms and regulates transcription of senescence-related genes (Panavas et al., 1998). In ethylene-sensitive flowers like carnation (*D. caryophyllus*), ABA accelerates flower senescence by increasing the endogenous production of ethylene (Ronen and Mayak, 1981). In contrast to the actions of ethylene and ABA, cytokinins delay senescence in vegetative and floral tissues (Van Staden et al., 1988). An inverse relationship between cytokinin content and senescence occurs in some flowers (Van Staden et al., 1988). Cytokinin content in roses (Mayak et al., 1972), carnation (Van Staden and Dimalla, 1980), and *Cosmos sulfurous* (Saha et al., 1985) is greatest in young flowers and decreases during corolla opening and development. Rose varieties with longer vase lives have been reported to contain more cytokinins than those with shorter vase lives (Mayak and Halevy, 1970). Results from exogenous application of cytokinins in vase solutions have been variable (Baker, 1983). Cytokinin application delayed senescence in carnations (Upfold and Van Staden, 1990),

roses (Mayak and Halevy, 1974), *Gerbera* sp. (van Meeteren, 1979), and petunia (*Petunia x hybrida*; Taverner et al., 1999), but the response depended on the type and concentration of cytokinin and the stage of flower development.

Interactions between cytokinins and other hormones during senescence have been less studied. Cytokinin applications to carnation flowers delay senescence and are associated with reduced ethylene biosynthesis and decreased sensitivity to ethylene (Cook et al., 1985). A study suggested that ethylene production during petunia senescence promotes cytokinin degradation and inactivation by *O*-glucosylation (Taverner et al., 1999). The sensitivity of flowers to ethylene increases as they mature, and this sensitivity change also has a role in the initiation of senescence (Woodson and Lawton, 1988).

Until very recently, no genes involved in cytokinin biosynthesis had been identified from plants (Sun et al., 2003). Plants with altered cytokinin content have been generated by manipulating cytokinin biosynthetic gene, *ipt* (Medford et al., 1989). The *ipt* gene encodes isopentenyl transferase, an enzyme that catalyzes the condensation of dimethylallylpyrophosphate and 5′-AMP to isopentenyladenosine (iPA) 5′-phosphate. This is assumed to represent a rate-limiting step in cytokinin biosynthesis because the introduction of the *ipt* gene into plants results in increased accumulation of many forms of cytokinins (Morris, 1995). Very low increases in endogenous cytokinin content of transgenic plants have been associated with pleiotropic effects including inhibition of root growth, stunted shoots, reduced apical dominance, increased stem diameter, and retarded leaf senescence (Schmulling et al., 1999). An approach to target the expression of *ipt* to senescing tissues with the promoter from SAG12, a senescence-associated gene from *Arabidopsis*, demonstrated a direct effect of cytokinins on plant senescence (Gan and Amasino, 1995). Numerous plants transformed with SAG12-IPT have significant delays in leaf senescence (Gan and Amasino, 1995; Jordi et al., 2000; Zhang et al., 2000; McCabe et al., 2001). Studies with these plants have confirmed that changes in cytokinin content impact the initiation of leaf senescence, nitrogen partitioning, photosynthetic enzyme activities, and chlorophyll degradation during senescence (McCabe et al., 2001). Sustained cytokinin content also enhances flooding and drought tolerance (Dervinis, 1999; Zhang et al., 2000). Although there are reports of SAG12-IPT plants with enhanced flower longevity (Zhang et al., 2000; Schroeder et al., 2001), there has been no investigation of the effects of endogenous cytokinin levels on the regulation of flower senescence beyond these phenotypic observations. Chang et al. (2003) used transgenic petunias expressing the P_{SAG12}-*IPT* chimeric gene to determine the effects of increased cytokinin levels in petals on flower senescence and to investigate the interactions between cytokinin accumulation and ethylene production, ethylene sensitivity, and ABA accumulation. Comparisons with nontransformed control plants demonstrated interactions between these signaling molecules that resulted in significantly delayed floral senescence. Identification of cytokinin receptors and cytokinin-responsive genes have increased our understanding of cytokinin signaling and interaction between cytokinin and other signals in recent years (Brenner et al., 2005; Ferreira and Kieber, 2005; Rashotte et al., 2005). Recent analysis of transgenic petunia plants with upregulated cytokinin biosynthesis showed that the flowers were less sensitive to exogenous ethylene and required longer treatment with ethylene to induce endogenous ethylene production and corolla senescence, and to upregulate senescence-related genes (Chang et al., 2003). Raised cytokinin content in plants has also been linked to improved tolerance of stress (Clark et al., 2004). Elsewhere, ethylene production in petunia corollas has been shown to promote senescence by

stimulating the metabolism of cytokinin bases and ribosides, thereby reducing cytokinin activity (Taverner et al., 1999).

4.8 Abscisic acid

Abscisic acid (ABA) is generally known as a strong growth inhibitor and a senescence-stimulating factor, but it also controls stomata closure in certain plants. In vegetative tissues, ABA appears to be involved in the response and adaptation of plants to environmental stresses, especially in drought, salinity, and cold conditions (as may occur in storage conditions of cut flowers). It has also been proposed that under water stress, turgor pressure declines and it results in an increase in cytosolic and apoplastic ABA levels (Patterson, 2001). This increase leads to (a) the closure of stomata to avoid further water stress and (b) the induction and accumulation of compatible solutes, such as proline, for water stress tolerance (Shen and Ho, 1997). Exogenous applications of ABA can serve also to increase the cold hardiness of plants. Provision of exogenous ABA in the vase solution effectively reduces vase solution usage and extends flower life. The expression of ABA genes results in the formation of, among other gene products, LEAs (late embryogenesis abundant), which are neither enzymes nor storage proteins but rather serve to protect proteins and membranes from damages during water loss in the cytoplasm due to desiccation. Reid (1985) hypothesized that a gradient of auxin from the subtended organ to the plant axis maintains the abscission zone in a nonsensitive state. A reduction or reversal of the auxin gradient causes the abscission zone to become sensitive to ethylene. Consequently, senescence and abscission could be altered by factors like shading, low irradiance, high temperature or water stress, and poor nutrition, which alter auxin gradients by exposure to ethylene, and by stresses that enhance ethylene production.

4.9 Gibberellins and other plant growth regulators

Gibberellins (GAs) are a large family of diterpenoid compounds, some of which are bioactive growth regulators, controlling such diverse processes as germination, stem elongation, and flowering. Cytokinins, and in some cases, GAs (such as GA3, GA4, and GA7 in *Alstroemeria* cut flowers; Jordi et al., 1996) delay the loss of Chl, whereas ethylene and ABA enhance the rate of Chl loss; the application of BA and GAs together improved the postharvest quality of cut Asiatic and Oriental lilies. Jasmonic acid can induce senescence, while polyamines delay foliar senescence (Singh et al., 2005a, b). In waxflower, the foliage of cut flowers often desiccates before flowers on the same sprig become senescent, since leaves have relatively higher turgor and lower osmotic potentials than flowers, and are less elastic (Joyce et al., 2000). Brassinosteroids induce PCD and the formation of secondary walls (Kuriyama and Fukuda, 2002). Growth retardants (e.g., uniconazole, ancymidol, paclobutrazol) reduce shoot elongation normally through inhibition of GA biosynthesis. GA effectively retains Chl, while the addition of indole-3-acetic acid (IAA) in vase water has little effect on leaf yellowing, while kinetin delays it (van Doorn et al., 1992). Benzyladenine (BA) improved the vase life of anthurium, heliconia, and ginger (*Alpinia purpurata*) when applied as a spray or a dip, but inhibited that of bird-of-paradise (*Strelizia reginae*), beehive ginger (*Zinziber spectabilis*) and Uluhe fern curls (*Dicranopteris linearis*), bamboo orchid (*Arundina bambusifolia*), and cut leaves of the fern lycopodium

(*Lycopodium cernuum*) (Paull and Chantrachit, 2001). Jasmonic acid and methyl jasmonate, natural plant growth regulators, are involved in plant defense responses, exhibit direct antifungal activity as well as increase numerous antifungal compounds in plant tissues when applied exogenously. They also activate many inducible genes leading to the synthesis of secondary plant products that function as antimicrobial compounds (Meir et al., 1998).

4.10 Identification and classification of senescence-related genes

Recent molecular studies have confirmed that senescence and ripening are accompanied by changes in gene expression. Utilizing differential screening and subtractive hybridization techniques, a number of cDNAs that are upregulated during senescence have been cloned. Genes that exhibit enhanced expression during senescence have been cloned from the leaves of *Arabidopsis*, asparagus, *Brassica napus*, barley, maize, radish, and tomato (Smart, 1994; Buchanan-Wollaston, 1997; Nam, 1997; Weaver et al., 1997; Quirino et al., 2000). Differential screening of senescence petal cDNA libraries and PCR-based differential display techniques have been utilized to identify genes that are upregulated during senescence of carnation and daylily flowers (Woodson et al., 1993; Woodson, 1994; Valpuesta et al., 1995; Guerrero et al., 1998; Panavas et al., 1999).

Most of the genes that have been identified as senescence-related are expressed at basal levels in nonsenescing tissues (green leaves and young flowers) and increase in abundance during senescence. A smaller number of SR genes are only detectable in senescing tissues and represent senescence-specific genes. An even smaller set of genes have been identified that have high levels of expression early in development, decreased expression in young maturing tissue, and increased expression at the onset of senescence. Genes that fit within this class have only been identified in vegetative tissues and represent genes that have a similar role in multiple stages of development like germination and senescence (Lohman et al., 1994; Smart, 1994; Buchanan-Wollaston, 1997).

Weaver et al. (1998) shows the patterns of expression of a selected group of SAGs (senescence-associated genes) during age-related senescence of *Arabidopsis* leaves. Most of these genes exhibit basal levels of expression in green nonsenescing tissues. Within this broad classification, genes are differentially regulated, with some increasing in abundance gradually as the leaf matures and others increasing more abruptly at various stages of leaf development. Only *SAG12* and *SAG13* show senescence-specific expression. Among the senescence-specific genes, *SAG13* is detected before any visible signs of leaf senescence and, as such, may be responsible for initiation of the senescence process, while *SAG12* is expressed after the leaf is visibly yellowing.

Many of the genes that have been identified as senescence-related are identified from a particular plant organ, and it is not known whether they are expressed in other senescing organs or during other developmental processes. The expression of a number of SAGs was investigated in roots, stems, flower buds, and mature flowers of *Arabidopsis* (Quirino et al., 1999). Expression of *SAG12, SAG13, SAG25, SAG26*, and *SAG29* was not detected in any nonsenescing tissues but was detected in both senescing flowers and leaves, indicating a common molecular regulation of senescence in vegetative and floral tissue. Some of the SAGs show low levels of expression in multiple tissues with upregulation in senescing

leaves and flowers (*SAG23*) or upregulation detected only in senescing leaves (*SAG28* and *SAG24*). *SG27* shows strictly leaf senescence-specific expression (Quirino et al., 1999).

Buchanan-Wollaston group has also identified a number of SR genes from *B. napus* utilizing both differential library screening and subtractive hybridization (Buchanan-Wollaston, 1994, 1997; Hanfrey et al., 1996; Buchanan-Wollaston and Ainsworth, 1997). It is not known whether any of these genes are upregulated during petal senescence, but similar to the *Arabidopsis* SAGs, they show differential patterns of expression during the development of the leaf. The homolog of the *Arabidopsis* (*SAG12*) gene has been cloned in *B. napus* and shows a similar senescence-specified expression after the onset of leaf senescence (Noh and Amasino, 1999b).

There is significant evidence that petal senescence in some flowers is a genetically programmed event that requires de novo protein synthesis and transcription of few genes (Woodson, 1987, 1994). In vitro translation of carnation petal mRNAs has revealed that the initiation of petal senescence is associated with increases in certain mRNAs (Woodson, 1994). Differential screening of a cDNA library from senescing carnation petals has identified nine cDNAs that represent unique senescence-related mRNAs. A cysteine protease (*DCCP1*) and three ACC-synthase (*DCACS1*, *DCACS2*, and *DCACS3*) cDNAs were identified from carnation petals using reverse transcriptase-polymerase chain reaction (RT-PCR) (ten Have and Woltering, 1997; Jones and Woodson, 1999). Only *SR139* and *DCCP1* transcripts are detected in preclimacteric petals. Most of the SR genes are detected in petals at 5 days after harvest, corresponding to the first detectable ethylene production from the petals. Eight of the eleven SR genes from carnation are flower specific, while low levels of *SR139*, *SR123*, and *DCCP1* are detectable in leaves (Woodson et al., 1992, 1993). Identifying the function of additional flower-specific SR genes will help to identify differences between the regulation of vegetative and floral senescence.

4.11 Regulation of SR gene expression by stress

Little is known about the transcriptional regulation of the SR genes other than that their mRNAs accumulate in one or more senescing tissues. While senescence is under developmental regulation, it can be accelerated by certain environmental stresses (Nooden, 1988). A few of the genes that have recently been identified as senescence related were previously identified based on increased transcription, following exposure to various stresses including drought and darkness (Park et al., 1998; Weaver et al., 1998). Studies by Weaver et al. (1998) have shown that a number of the SAGs from *Arabidopsis* are induced by stresses. Exposing excised leaves to darkness is one of the strongest inducers of SAG transcript accumulation. While detaching leaves in the light did not result in leaf chlorosis, it did result in the enhanced expression of most SAGs (Weaver et al., 1998). Only about half of the SAGs tested showed increased expression following dehydration treatments. In general, these responses were influenced by the developmental stage of the leaves with strongest induction in older leaves and no effect or only moderate induction detected in young leaves (Weaver et al., 1998).

Physiological and biochemical changes such as decline in photosynthetic rate and chlorophyll content have been used to identify stages of senescence, but artificial methods such as detachment and darkness that rapidly induce senescence have become popular models for studying leaf senescence (Thomas and Stoddart, 1980). While the

detachment of leaves in the dark accelerates senescence and serves as a means of coordinating the senescence process between leaves, its use as a model system for natural age-related senescence should be approached with caution. Following the identification of three cDNAs that showed increased expression in dark-treated leaves, experiments revealed that only one of the three showed enhanced expression during natural senescence (Becker and Apel, 1993). Only four of seven genes identified from *Arabidopsis* as dark-induced also showed enhanced expression in naturally senescing leaves (Park et al., 1998).

There are many common molecular events occurring among senescing tissues. The degradation and remobilization of cellular constituents is predominant during senescence and, correspondingly, the activities of hydrolytic enzymes and their mRNAs increase. A number of SR genes have also been identified that encode products with homology to PR proteins. While it is not known what the role of these proteins is in senescence, it appears that they may serve a protective role similar to their role during the defense response. These patterns of expression indicate that throughout plant development, common molecular mechanisms are regulated by the same genes in multiple tissues. A few of these genes have been identified as having leaf, flower, or fruit senescence-specific expression. Of these genes, many encode different isoforms of the same enzyme, which may be differentially regulated within the plant organs. Similar to fruit ripening, the developmental regulation of germination and senescence also share common molecular mechanisms. This is especially evident when investigating the expression of genes involved in protein and lipid degradation and remobilization.

While differential cDNA screening, differential display and cDNA subtraction have identified a number of senescence-related genes, the expression of most genes has not been investigated in flowers, leaves, and fruits. The use of enhancer trap lines in *Arabidopsis* has resulted in the identification of over 100 lines that have reporter gene expression in senescing, but not in nonsenescing tissues (He et al., 2001). This technology starts to reveal the complexity of the network of senescence-regulated pathways, and will allow for the identification of many additional SR genes. The identification of senescence-specific promoter elements (Noh and Amasino, 1999a) and the generation of mutants and transgenic plants will help us to better understand the regulation of SR genes during senescence. DNA microarrays will allow temporal and spatial expression patterns to be determined for hundreds of genes involved in senescence. These technologies will lead to an increased understanding of the initiation and execution of senescence, which will allow us to increase vase life and horticultural performance of ornamentals, increase yield in agronomic crops, and decrease postharvest losses of fruits and vegetables.

4.12 Sugar status and cut flower senescence

Sugar solutions are well known for their ability to improve postharvest quality and extend the vase life of cut flowers, although the hypothesis of a sole sugar starvation or sugar accumulation signal in inducing petal senescence has not been validated (van Doorn, 2004). Signals from the lack of, the abundance of, or the metabolism of sugars (i.e., sugar signals) probably form part of a complex array of exogenous and endogenous signals that initiate senescence, and there is evidence that in carnation petals sucrose decreases ethylene responsiveness (Verlinden and Garcia, 2004), and complex interactions occur between sugar- and ethylene-signaling mechanisms that are tissue dependent (Iordachescu and Verlinden, 2005). In

addition, the cytokinin-mediated delay of senescence in leaves has been linked to activity of invertases (Balibrea Lara et al., 2004) (enzymes that hydrolyze sucrose into glucose and fructose), and since petals share many molecular and hormonal features with leaves, this may also prove to be the case for flowers. The complexity of interactions known to occur between sugars and plant hormone pathways in plants has been outlined in a review (Gibson, 2004). Sugars are also known to modulate plant growth and development (Rolland et al., 2002), and in flowers the breakdown of long-chain carbohydrates (e.g., fructan and starch) is one of the main drivers of petal opening (Bieleski, 2000). Functional analyses of sucrose transporter proteins have shown that these proteins load sucrose into the sieve elements of source organs, and that their activity is critical for the normal growth, development, and reproduction of *Arabidopsis* plants (Gottwald et al., 2000). The activity of petal-specific phloem-loading sucrose transporters has been postulated to achieve the rapid transition from sink to source in relation to phloem movement (Bieleski, 2000), as floral tissues shift from mature to senescence states. Sugar signaling has also been implicated in the regulation of gene expression in plants (van Meeteren et al., 2000), and sugar-feeding treatments alter the expression of senescence-related cysteine protease and β-galactosidase genes in *Sandersonia* cut flowers (O'Donoghue et al., 2005). The integration of sugar or polyols-containing pulsing solutions into postharvest regimes is effective for maintaining quality and delaying the onset of senescence in gladiolus (Arora and Singh, 2006) and many other cut flowers. Experimentation suggests that sugars have a role not only as an energy source but also in regulating gene expression.

4.13 Membrane permeability

A consistent feature of senescence is the loss of differential permeability of cell membranes (Thompson, 1988). Deterioration of cellular membranes causes increased membrane permeability, loss of ionic gradients, and decreased function of key membrane proteins (e.g., ion pumps) (Paliyath and Droillard, 1992). Changes in the properties of membranes, such as increases in microviscosity, alterations in saturation/desaturation ratios of fatty acids, and peroxidation of lipids, are known to occur during petal senescence, with a causal link to reactive oxygen species, which are often elevated as a result of stress and have been implicated in the progression of petal senescence. Membrane deterioration is commonly associated with progressive decreases in membrane phospholipid content through phospholipase activity. Increased lipase (lipolytic acyl hydrolase) and lipoxygenase activity has been linked to the onset of membrane leakiness in carnation (Hong et al., 2000) and rose (Fukuchi-Mizutani et al., 2000), respectively, but a loss of membrane function in *Alstroemeria* occurs without increased activity of lipoxygenase (Leverentz et al., 2002), suggesting that loss of membrane integrity can be achieved in a number of ways. In petals, phosphatidylcholine and phosphatidylethanolamine make up 75% of the membranes' phospholipids (Borochov and Woodson, 1989). A senescence-induced lipase with lipolytic acyl hydrolase activity has been identified from carnation flowers (Hong et al., 2000). The abundance of the lipase mRNA increases just as carnation petals begin to in roll and is enhanced by treatment with ethylene. Understanding the cause of membrane breakdown in senescing tissues also has implications for signal transduction chains, as the components of these chains are often associated with membranes. These aspects are addressed in Chapter 9.

4.14 Cell wall changes during wilting

Cell walls are composed of a strong flexible network of cellulose surrounded by a hydrated matrix of acidic polysaccharides (pectin), hemicelluloses, structural glycoproteins, and enzymes (Cosgrove, 2000). Cell walls provide protection and structure for individual cells and must accommodate rapid cell or tissue-specific expansion during petal opening. The onset of senescence-associated wilting of floral organs has been temporally linked with modifications of the cell wall in carnation (Devetten et al., 1991), and recent work on *Sandersonia* also supports this view (O'Donoghue et al., 2005). Anatomical changes to flower petal cells suggest that cell walls swell or break down as the internal mesophyll cells become separated from each other and collapse during petal expansion and subsequent senescence (O'Donoghue et al., 2002; Wagstaff et al., 2003). The loss of cell order that occurs during petal senescence is often accompanied by an increase in activity of cell wall hydrolases, depolymerization of hemicelluloses, and loss of neutral sugars, particularly galactose and arabinose (O'Donoghue et al., 2002). *Sandersonia*, in particular, loses nearly 50% of cell wall galactose as flowers begin to wilt, accompanied by a marked increase in β-galactosidase (O'Donoghue et al., 2002, 2005). A putative role for cell wall β-galactosidases in ripening fruit tissue is the degradation of pectin-associated galactan, which increases wall porosity and enables access of other cell wall–modifying enzymes (Brummell and Harpster, 2001). β-Galactosidase gene expression, enzyme activity, and galactose loss from the cell walls of *Sandersonia* petals may be associated with the onset of wilting (O'Donoghue et al., 2005), but the theory of increased wall porosity may not apply in these flower petals. The senescence-associated expression of *Sandersonia* β-galactosidase genes occurs in concert with protease genes and is delayed after sugar-feeding treatment, which also delays the onset of petal wilt (O'Donoghue et al., 2005). *Sandersonia* β-galactosidase genes are also differentially expressed after water stress treatments. Expansins are noncatalytic enzymes that are believed to disrupt the hydrogen bonding that links cellulose microfibrils with matrix polysaccharides. Two roles for expansins in plants have been proposed: enabling cell walls to expand in response to increased turgor pressure within the cell (Cosgrove, 2000) and cell wall disassembly during tissue senescence (Brummell et al., 1999). The role of expansins in petal senescence is less clear, one hypothesis is that expansins function to destroy cell wall integrity by indiscriminate binding (Gookin et al., 2003). In petunia, downregulation of α-expansin gene (*PhEXP1*) produced plants with flowers of a smaller size (Zenoni et al., 2004). The petals had a smaller epidermal cell area and altered cell wall morphology and composition. The reduced cell wall thickness in transgenic plants was accompanied by a reduction in crystalline cellulose (Zenoni et al., 2004). The expansins play a key role in regulating cell expansion in petunia petals by coordinating cellulose synthesis, deposition, and spatial organization in relation to other polymers and protein constituents of the cell wall (Zenoni et al., 2004).

4.15 Changes in nucleic acids and proteins

While senescence is a degradative process, this degradation is not merely the result of increased rates of protein turnover and decreases in the synthesis of proteins and RNA. Although general decreases in total protein and RNA levels are observed in senescing floral and foliar tissues, specific proteins and mRNAs have been found to increase (Borochov

and Woodson, 1989; Arora and Singh, 2004). Experiments with inhibitors of protein and RNA synthesis have demonstrated that senescence is a genetically programmed process that requires the selective activation of specific RNAs and proteins, and does not merely result from the inhibition of cellular metabolism by declining rates of protein and RNA synthesis. These inhibitor studies have also suggested that transcription and protein synthesis in organelles is not central to the regulation of senescence. In support of the nuclear regulation of senescence, nuclear genes have been found to encode almost all of the mRNAs found to increase during senescence (Nooden, 1988). While the later stages of ripening resemble senescence, the entire process represents more of an interaction between degradative and synthetic processes. In contrast to senescing flowers and leaves, protein levels in fruits remain constant or increase slightly during ripening (Brady, 1988). Specific mRNAs and proteins that increase during ripening have also been identified (Gray et al., 1992). These will be referred to as senescence-related (SR) genes.

4.15.1 Plant cysteine proteinases

Proteolysis in plants is a complex process involving many enzymes and multifarious proteolytic pathways in various cellular compartments, with cysteine proteinases playing an essential role. Their share in total proteolysis depends on the kind of plant and its organ. It amounts up to 30% of total proteolytic activity in mature nonsenescing organs. However, the activities of cysteine proteinases respond dramatically to different internal and external stimuli, and in some cases, they rise to 90% of the total proteolytic activity (Wisniewski and Zagdanska, 2001). They are involved in protein maturation, degradation, and protein rebuilt in response to different external stimuli, and they also play a housekeeping function to remove abnormal, misfolded proteins. In each case, the proteolysis by cysteine proteinases is a highly regulated process.

4.15.2 Proteolysis regulates metabolic processes within the cell

Proteolysis is an indispensable process in all living organisms. A continual turnover of proteins removes functionally impaired proteins (due to biosynthetic errors, improper folding, thermal denaturation, oxidative damage (Arora et al., 2002), which if left unchecked, may restrict metabolic activities and jeopardize a cell's integrity. Proteases also recycle essential amino acids and are important in the recovery of valuable nutrients. Proteases regulate metabolic pathways and developmental programs by affecting the rapid turnover of rate-limiting enzymes and key regulatory proteins (Clarke, 2005). Proteolytic cleavage is thought to play a significant role in the senescence of flowers because expression of protease genes is one of the earliest senescence-related gene changes to be identified (Eason et al., 2002). Upregulated expression of protease genes, raised enzyme activity, and a decline in soluble protein levels occur consistently during senescence, both of ethylene-sensitive (Wagstaff et al., 2002; Jones et al., 2005) and ethylene-insensitive flowers (Eason et al., 2002; Arora and Ezura, 2003; Arora and Singh, 2004). Regulating the senescence-associated activity of proteases may be achieved with different molecular strategies. First, the interaction between proteases and their inhibitor proteins have been linked to modulation of cell death processes in plants (Sin and Chye, 2004). In certain cut flowers (*Sandersonia* and *Iris*), chemical inhibition of protease action delays the onset of senescence (Pak and van Doorn,

2005), and the accumulation of cysteine protease mRNAs in senescing carnation flowers is associated with a corresponding decline in protease inhibitor mRNA (Sugawara et al., 2002), indicating that inhibitor proteins may play a role in regulating senescence-associated protease activity in flowers. Second, proteases have been shown to be localized to the plant vacuole (Sin and Chye, 2004; Lam, 2005), and both posttranslational modification and subcellular localization provide the cell with a means to regulate protease activity. Another avenue for extending the display life of cut flowers through modification of proteolysis is the downregulation of senescence-associated cysteine proteases. In broccoli, this strategy has produced transgenic plants with delayed postharvest senescence (Eason et al., 2005) and may offer an alternative strategy for extending vase life of cut flowers. It has been reported that a single daffodil flower will cause an increase in the vase life of other flowers (van Doorn et al., 2004). The active compound in the mucilage of daffodil flowers, which delays tepal senescence in cut *Iris* flowers, is narciclasine, an alkaloid known to inhibit protein synthesis (van Doorn et al., 2004). This also provides a new strategy for postharvest vase life extension, if specific protease inhibitors can be developed in the future for use in vase solutions.

4.15.3 Genes involved in protein degradation

Decreases in total proteins during senescence result from increases in proteolytic enzyme activity and decreases in protein synthesis (Brady, 1988). The degradation of proteins and remobilization of amino acids to developing tissues is the predominant metabolic process during senescence. Cysteine proteases are believed to be the main proteases involved in general protein hydrolysis, and recently, a number of cysteine protease have been identified from senescing leaves, senescing flowers, and ripening fruit.

Of those cysteine proteases identified from senescing tissues, most share sequence homology with γ-oryzain from rice, a cysteine protease that has been implicated in the mobilization of reserve proteins during seed germination. These include *SAG2*, *See1*, *LSC7*, *SENU2*, and *SENU3*. The expression patterns of these five genes are similar, with low levels of expression in young leaves and increased expression during senescence (Buchanan-Wollaston, 1997; Weaver et al., 1998). Both tomato cysteine protease, *SenU2* and *SenU3* and *See1*, from maize also show patterns of upregulation during seed germination, indicating that these proteases may play similar roles in protein degradation during germination and leaf senescence (Smart et al., 1995; Drake et al., 1996). While common to germination and leaf senescence, the *SENU2* and *SENU3* transcripts were not upregulated during fruit ripening (Drake et al., 1996). *SAG12*, which encodes a papainlike cysteine protease, is one of the few SR genes to the display senescence-specific regulation. *SAG12* mRNAs are not detectable in roots, stems, green leaves, or young flowers, but increase in abundance in senescing petals as well as leaves (Lohman et al., 1994; Quirino et al., 1999). This senescence-specific expression suggests that the *SAG12* protease might play a key role in the large-scale increases in protein degradation during senescence.

The dismantling of the chloroplast, which contains greater than 50% of the leaf's total protein, is a prominent process in leaf senescence (Thomas and Stoddart, 1980). While many SR genes have been identified as proteases, only one of these has been found to be localized to the chloroplast (*Erd1*; Lohman et al., 1994). Transcript levels of clp protease have been reported to increase during leaf senescence, but protein levels were found to decline,

suggesting that the clp protease does not play a primary role in the programmed disassembly of the chloroplast during senescence (Weaver et al., 1999). A study by Guiamet et al. (1999) has reported that the chloroplast of senescing soybean leaves excrete plastoglobuli-containing constituents of the chloroplast. The dismantling of these chloroplast components then occurs outside the chloroplast where SR proteases are localized.

Similar to leaf senescence, protein degradation has been demonstrated to be a major part of petal senescence and the remobilization of N to the developing ovary (Nichols, 1976). A few of the SR cysteine proteases have been shown to be upregulated in both leaves and petals (*DCCP1*, Jones et al., 1995; *Peth1*, Tournaire et al., 1996; *SAG12*, Quirino et al., 1999; *GgCyP*, Arora and Singh, 2004). Large increases in proteolytic activity during the senescence of the ephemeral flower, daylily, have been well documented, and this proteolytic activity was correlated with increases in the expression of two cysteine protease genes (*Sen11* and *Sen102*) during the senescence of petals (Valpuesta et al., 1995; Guerrero et al., 1998; Stephenson and Rubinstein, 1998). In contrast to the cysteine proteases from carnation and petunia, transcripts are not detectable in young daylily flowers (buds) and the level of transcript does not increase in senescing leaves (Guerrero et al., 1998). Both daylily cysteine proteases appear to be flower senescence specific. Arora and Singh (2004) studied the changes in protein content and protease activity in the petals of ethylene-insensitive gladiolus flowers, during development and senescence. There was a dramatic upregulation in the expression of *GgCyP* at the incipient senescent stage of flower development, indicating that this gene may encode an important enzyme for the proteolytic process in gladiolus. The gladiolus cysteine protease gene appears to be flower senescence specific.

4.16 Molecular strategies of extending cut flower life

Conventional breeding is still a practical form of increasing the number of flowering buds, extending the longevity of an inflorescence and improving its postharvest performance, as has been demonstrated in *Lilium* (van der Meulen-Muisers et al., 1999). Many of the molecular mechanisms underlying senescence, and the respective genes involved in protein degradation, nucleic acid and chlorophyll breakdown, and lipid and nitrogen remobilization have been extensively covered in other reviews (Buchanan-Wollaston, 1997; Gan and Amasino, 1997). An understanding of these mechanisms is vital to the use of molecular techniques to clone genes of interest to reverse, for example, through antisense technology, the detrimental effects of senescence, aging, or PCD. Maternal inheritance of herbicide resistance via chloroplast engineering, or hyperexpression of lethal insecticidal proteins (other than the Bt (*Bacillus thuringiensis*) gene product) provides new genetic solutions to biocontrol of infectious agents in development of phytosanitary control.

PCD in plants is well documented, and it is not only synonymous with senescence (leaf and flower), but is also a fundamental part of a plant's adaptation to stresses, such as reactive oxygen species. The termination of a flower involves two overlapping mechanisms (Rubinstein, 2000), one being petals that abscise before the majority of their cells initiate a cell death program, and where abscission may occur before or during the mobilization of food reserves to other parts of the plant. In the second, the petals are more persistent, and cell deterioration and food remobilization occur while the petals are still part of the flower. One way of countering the effects of pathogen-induced PCD is through the use of caspase inhibitors in the cut flower medium (Richael et al., 2001).

Numerous ethylene-insensitive mutants, such as *Arabidopsis thaliana* etr1-1 or ein-2, or never ripe tomato mutants exist (Zacarias et al., 1999). Flowers could be engineered to produce reduced levels of ethylene by introduction of an antisense ACC-oxidase transgene, as occurs in tomatoes (FLAVR SAVR®), driven by a flower or senescence-specific promoter (John et al., 1995; Wilkinson et al., 1997; Bleecker et al., 1998; Zacarias et al., 1999). Transgenic fruits containing ACC deaminase and antisense ACC synthase, ACC oxidase, and polyphenol oxidase have been produced, the first three reducing ethylene production and slowing ripening, the last reducing browning of damaged tissue (Flores et al., 2001).

When the endogenous cytokinin status is manipulated through transgenic intervention, a stay-green phenotype can be obtained, as occurred in the fusion of ipt, an Agrobacterium gene encoding a limiting step in cytokinin biosynthesis, to an *Arabidopsis* See (SAG12) promoter (Gan and Amasino, 1997). Greenness can also be altered (delay in leaf senescence) by downregulating the production of a senescence-promoting hormone, as seen in tomato plants in which ethylene biosynthesis is inhibited by antisense suppression of the gene for ACC oxidase (John et al., 1995).

4.16.1 Genetic manipulation

Within flower species and cultivars, there is great variability in ethylene sensitivity (of the flowers). This implies that breeding toward less sensitive flowers is possible. In fact, almost all modern carnation cultivars are much less sensitive to ethylene than the cultivar "White Sim" that has been used over the years to study ethylene-induced senescence. In many flowers, breeding programs aiming at a better vase life may (unintentionally) target the ethylene biosynthesis and perception processes. To the best of our knowledge, breeding of plants that are highly insensitive to ethylene in fully developed flowers has not been successful so far. A few attempts to produce plants with ethylene insensitive flowers, or with low production of endogenous ethylene, have been performed (Onozaki et al., 2001). Facilitated by the detailed knowledge of ethylene production and perception in plants, several attempts have been made to produce plants with prolonged flower life by genetic transformation. The first experiments in this line of work were based on ACC oxidase (ACO), the last enzyme in the ethylene biosynthetic pathway. Savin et al. (1995) transformed carnation with a construct in which an antisense sequence of the carnation ACO gene was placed under control of a constitutive promoter. This resulted in a few plants with dramatically reduced ethylene production during flower senescence, and with flower longevity of 8–9 days for cut flowers compared to 5 days for the nontransformed flowers. An attempt to use this technique in *Begonia* failed (Einset and Kopperud, 1995). Transgenic plants were obtained, but they did not show prolonged flower life. The ornamental *Torenia fournieri* has been successfully transformed using both sense and antisense ACC-oxidase gene constructs (Aida et al., 1998). The transgenic plants showed slight but significant enhancement of flower longevity. Carnations with reduced ACC-synthase activity using a cosuppression technique were produced at Florigene (Michael et al., 1993; patent no. WO9635792, transgenic carnations exhibit prolonged postharvest life). Transgenic flowers had a much longer vase life than wild type, but also showed problems related to a decreased resistance to fungal pathogens. Till now, these products have not yet entered the market. The first attempt to block the function of the ethylene receptor was done by Hua et al. (1995) using a mutated *Arabidopsis* ers gene. Transgenic *Arabidopsis* plants showed strong

tolerance to endogenous ethylene. Much more work has been done using the mutated dominant ethylene-resistance gene *etr1-1* from *Arabidopsis*. Early experiments with this gene were done in *Petunia* (Wilkinson et al., 1997; Clark et al., 1999; Gubrium et al., 2000) and continued by Celvenger et al. (2004). In these very thorough experiments, a CaMV35S:: etr1-1 construct was used, resulting in constitutive expression of the *etr1-1* gene. The results showed that etr1-1 from *Arabidopsis* conferred ethylene insensitivity to the plants, but also that constitutive expression of the gene gave some additional effects that, though expected, are unacceptable from a grower's point of view. The effect of the transformation proved to be dependent on the genetic background and on the temperature the plants were kept.

Typical effects observed in these plants were poor root development of cuttings, lower seed weight, less efficient seed germination, less efficient rooting and delayed growth of seedlings, slightly faster flowering, enhanced ethylene production in pollinated flowers, delayed flower abscission, delayed fruit ripening, and delayed flower senescence.

Delayed flower senescence was seen both in the presence and absence of ethylene, and both in pollinated and nonpollinated flowers. From a grower's point of view, the most interesting of these effects is the delayed senescence and abscission of flowers; the poor rooting ability of cuttings is the most unacceptable. Most of these effects can be explained by the role ethylene plays at the different developmental stages. The enhanced ethylene production in flowers probably is due to the uncoupling of the feedback from the plant's reaction to pollination.

Shaw et al. (2002) have achieved ethylene-insensitive *Petunia* using a mutated ers homolog from *Brassica oleracea* (boers). The results from this experiment resemble those mentioned above, except for a higher mortality, due to a higher susceptibility to fungal diseases. This is not a surprising result, as ethylene is known to play a role in defense against pathogens. Also, these plants showed prolonged flower longevity. The ornamental *Nemesia strumosa* has also been transformed for enhanced flower longevity by altered ethylene perception controlled by a constitutive promoter (Cui et al., 2004). In this case the plants were transformed with a mutated etr1-homolog from melon. The gene in question contained a mutation resembling that of etr1-1 from *Arabidopsis*. Wilkinson et al. (1997) suggested the use of tissue-specific promoters in order to avoid the unintended side effects due to the use of the constitutive CaMV35S promoter. Bovy et al. (1999) followed this recommendation when they used a flower-specific promoter from *Petunia* (fbp1) in their attempt to transform carnation with etr1-1. The resulting plants showed strong insensitivity to ethylene without the unwanted side effects of earlier experiments, though not all agronomical aspects were investigated.

The gene construct used in the latter experiment has also been used in the transformation of *Campanula carpatica*. A very effective method for transformation and regeneration of this species has been developed (Sriskandarajah et al., 2001; Frello et al., 2002), and a number of transformed plants have been obtained and transferred to soil (Sriskandarajah et al., 2004). Another line of experiments have been carried out in *Petunia* by Chang et al. (2003). They used a construct (Psag12-IPT) containing a promoter from a senescence-associated gene coupled to a cytokinin biosynthetic gene from *Agrobacterium tumefaciens* to control the cytokinin production of senescing flowers. Several simultaneous effects were seen: the cytokinin level was enhanced, and this was accompanied by a delay in ethylene production and by enhanced ethylene tolerance of the flowers of the transgenic

plants. In concert, these changes gave a dramatic effect: the flower longevity was prolonged with approximately 100% in nonpollinated flowers and approximately 450% in pollinated flowers.

4.17 Breeding/genetic modification to improve postharvest quality

To date, use of gene transfer technology to delay flower senescence has highlighted the need for tightly regulated transgene expression to avoid affecting other nontarget developmental processes, particularly in the modification of plant hormone levels (e.g., poor rooting and lower disease resistance in ethylene-insensitive plants) (Clark et al., 1999; Shaw et al., 2002). Thus, the need for tissue-specific promoters is paramount for exploiting this avenue of crop development in commercially important cultivars. Alternatively, modifying the expression of metabolic genes may produce satisfactory postharvest improvements without the need to alter hormone biosynthesis or perception, which may have pleiotrophic effects. Pollen sterility in flowers innately lowers pollen-induced senescence signals, and may make currently unsuitable flowers suitable for cut flower cropping without needing antiethylene treatments. The use of traditional breeding to select for genetic improvement of vase life may progress more rapidly as genetic markers for "long life" are identified and as gene transfer technologies provide a way to improve the postharvest characteristics of crops with low genetic diversity.

4.18 Future perspectives

There is little doubt that the molecular and genetic analyses of flower senescence made in the past 5 years have raised our awareness of the complex interactions that occur to regulate flower development and senescence. Genetic technologies have enabled scientists to search for senescence-related genes in plants often described as science models (e.g., *Petunia*, *Arabidopsis*), and then translate the data into other species to determine the functional significance of the expression of specific genes in specific tissues after harvest. Interactions between ethylene, cytokinin, sugars, and various hydrolytic enzymes are now known to differentially mediate the progression of flower senescence. The individual importance of each signal appears to be species specific and, in some instances, variety specific, and varies differentially between floral organs. The challenge for postharvest scientists is to identify a hierarchy of regulators or a specific pattern of events that progresses senescence for certain groups of flower species. Subsequent categorization of cut flowers based on their metabolism and sensitivities will enable targeted application of appropriate postharvest technologies.

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Chapter 5

Programmed Cell Death during Plant Senescence

Ajay Arora

5.1 Introduction

Programmed cell death (PCD) is now recognized as an important event in shaping plant organs (Jones and Dangl, 1996; Pennell and Lamb, 1997). In animal cells, programmed cell death has been defined as cell death that is a part of the normal life cycle of the organism, is triggered by specific physiological signals, and involves de novo gene transcription (Ellis et al., 1991). This distinction is important in differentiating developmental PCD from necrotic cell death that may occur as a result of injury or exposure to toxic substances. In plants, an important response to pathogenic attack is the hypersensitive response (HR) in which selected cells around the site of infection undergo PCD to prevent spread of the pathogen. Although the cellular features of HR-PCD show many similarities to developmental PCD, this area has been reviewed (Greenberg and Yao, 2004) and will not be dealt with in detail here. In plants, it is also important to distinguish between PCD and senescence, terms that have led to much controversy in this field and have been the focus (van Doorn and Woltering, 2004). In this chapter, PCD will be used to indicate cellular death as opposed to the death of whole organs or individuals. In some plant organs, the effect of PCD is particularly evident. Thus, whereas in animals sculpting of the fingers is often quoted as the classic example of the action of programmed cell death in organ development, in plants an obvious example is the prominent holes in the leaves of the house plant *Monstera* (Melville and Wrigley, 1969). In animal cells at least two forms of PCD have been described: apoptosis and autophagy. A number of cytological features describe apoptosis. These include nuclear condensation and marginalization, chromatin condensation, followed by fragmentation of DNA into nucleosomal units known as DNA laddering, and the formation of membrane inclusions known as apoptotic bodies. In animals, the apoptosed cell remains are finally engulfed by neighboring cells through phagocytosis and the cell corpse disappears (Cohen, 1993). In a few cases, PCD may be defined as truly autonomous as in the very tightly regulated cell death in *Caenorhabditis elegans* (Yuan and Horvitz, 1990). However, in many cases, signals external to the cell, such as changes in hormone levels, trigger PCD (Cohen, 1993). Once PCD has been triggered, a complex network of regulators is switched on involving increases in cytosolic calcium concentrations, an oxidative burst, and release of pro-PCD factors such as cytochrome *C* from the mitochondrion. Release of cytochrome *C* is regulated by a growing family of Bcl-2 proteins

that interact with the mitochondrial membrane, facilitating or inhibiting its release. These intracellular events activate a family of cysteine aspartate–specific proteases known as caspases, which are both regulators and the effectors of cell death. Caspases act on a plethora of targets initiating cell condensation, nuclear fragmentation, and DNA breakdown. The characteristic DNA laddering occurs as a result of cleavage at the nucleosome linker sites by DNase, which is activated both by the caspases and more directly by increases in cytosolic calcium levels (Peitsch et al., 1993). Autophagy in mammalian cells (Stromhaug and Klionsky, 2001) was originally associated with a response to starvation. It is characterized by the formation of vesicles-containing proteins and organelles that are transported to the lysosome. Known as autophagosomes, these vesicles have a short half-life (in the range of a few minutes), and their contents are then digested by the hydrolase-packed lysosomes to generate monomeric building blocks. The signaling pathways leading to autophagy are much less well-defined than those involved in apoptosis; however, heterotrimeric G proteins (Ogier-Denis et al., 2000) and type III phosphoinositide 3-phosphatases (Petiot et al., 2000) have been implicated. Interestingly, autophagy and apoptosis may not be completely independent mechanisms. Beclin 1, which interacts with Bcl-2 family of proteins, is associated with type III phosphoinositide 3-phosphatases, and may have a role in sorting proteins destined for the autophagosome (Kihara et al., 2001). Also, sphingosine, known to activate apoptosis, increases the activity of lysosomal proteases such as cathepsin B, which in turn are caspase activators (Ferri and Kroemer, 2001). Notably, the lysosomes appear to act upstream of the mitochondria, suggesting a regulatory role in apoptosis (Yuan et al., 2002). Few genes with sequence homology to those involved in animal PCD have been identified in plants despite the availability of genome sequences from representatives of the major taxonomic divisions of flowering plants, namely, *Arabidopsis* representing dicotyledonous plants (The Arabidopsis Genome Initiative, 2000) and rice representing the monocotyledons (Goff et al., 2002). Putative homologs include those for Bax inhibitor-1, an inhibitor of the proapoptotic Bcl-2 family member Bax (Kawai et al., 1999) involved in apoptosis, Beclin (Laporte et al., 2004) involved in autophagy, and dad-1 (Orzaez and Granell, 1997a), although doubt has been cast on the regulatory role of dad-1 (Kelleher and Gilmore, 1997). Although homologs of caspases have not been identified from the genome sequences (Lam and Del Pozo, 2000), plant metacaspase members of a related superfamily are found in plant genomes (Lam, 2004), and can trigger apoptosis-like cell death in yeast (Watanabe and Lam, 2005). In addition, caspase inhibitors inhibit plant PCD in several systems.

5.2 Pathways for PCD in plants

Built into the genome of almost all living organisms is a genetic program for cell suicide, or programmed cell death (PCD). However, although the function of this program is conserved among all organisms, the genetic pathways responsible for mediating and executing PCD might be very different among organisms such as bacteria, yeast, plants, and animals (Mittler, 1998). Furthermore, even within a single organism, many different pathways may control and mediate PCD (Beers and McDowell, 2001). In plants, many different cell death signals can activate PCD, and PCD can manifest itself in many different forms (Greenberg, 1996). Thus, for example, hypersensitive response-PCD and PCD of xylem vessels are initiated by very different signals and manifest themselves in very different forms (Dangl et al., 1996;

Fukuda, 1996). However, it is not entirely clear whether there is a master switch for PCD through which all pathways must go, or whether there are many different switches that are pathway specific.

In animals, the mitochondrion was found to play a central role in the activation of different PCD pathways. Studies in plants suggest that mitochondria may also play an important role in PCD in plants (Lam et al., 2001). However, it is too early to determine whether the mitochondrion is as central for PCD in plants, as it is for PCD in animals. The morphological and molecular differences that exist between the different PCD pathways in plants (i.e., developmental, abiotic stress-induced, disease symptoms, and the hypersensitive response-PCD; Mittler, 1998), as well as the selective inhibition of specific PCD pathways by overexpression of Bcl-2-like proteins or p35 in plants (Mitsuhara et al., 1999; Lincoln et al., 2002), may suggest that there are many switches for PCD activation in plants. It is possible that plants use PCD pathways that are similar to those found in animals (e.g., the mitochondrion); however, plants may use different proteins that share only a limited degree of homology with animal genes such as *bcl-2* and caspases (Kawai-Yamada et al., 2001; Lam et al., 2001).

5.3 Senescence and organ senescence as forms of PCD

S*enescence* or better the *senescence syndrome* refers to those degenerative processes leading to death that occur under the control of the plant (Nooden, 1988). It is a developmental program that guides the cell through an ordered schedule of events leading to the death of the cell/organ and serving, at the same time, a variety of functions selected by evolution to optimize plant survival (Granell, 1999). Implicit in the definition is the idea that despite being a degenerative process, it is still organized and remains under control of the cell.

Senescence is an active process that requires energy consumption, and part of the program is designed to supply it (Solomos, 1988; Buchanan-Wollaston, 1997). But program in this case also means that information on how to dismantle the cell is genetically driven and therefore emerges from the genes. While the programmed nature of senescence is strongly established, it is also becoming clear that more than one program or variations of the program may exist. For example, the general loss of membrane function accompanying senescence in *Alstroemeria* is not related to lipoxygenase activity or to the accumulation of lipid hydroperoxides that occurs in other plants (Leverentz et al., 2002). Microarray and proteomic analyses show commonalities and differences among the different senescence programs (Swidzinski et al., 2002, 2004).

5.4 Leaf senescence versus petal senescence

The process of senescence and cell death are clearly distinct at a physiological level because senescence, at least in leaves, can be a reversible process, whereas cell death is considered a terminal event (Thomas et al., 2003). Leaf senescence has been the focus of several reviews (Lim et al., 2003) and genomic-wide approaches to identify regulatory networks (Buchanan-Wollaston et al., 2003; Gepstein et al., 2003). The signals initiating the overall process of senescence are common to other PCD events. Reduction of ethylene signaling (Grbic and Bleecker, 1995) and upregulation of cytokinin production (Gan and Amasino, 1995) delay senescence, indicating that the levels of these two PGRs are involved. Elevated cytoplasmic

calcium is associated with leaf senescence in parsley (Huang et al., 1997), and calcium fluxes have also been implicated in two other leaf senescence systems (Chou and Kao, 1992; He and Jin, 1999). Thus, calcium signaling may play a role in leaf PCD although further data are needed. Cytologically, PCD has been charted in rice leaves undergoing induced (by dark treatment) or natural senescence (Lee and Chen, 2002). In this system, features noted were cytoplasm depletion, organellar breakdown, and expansion of the central vacuole, which at later stages contained inclusions possibly of chloroplast origin. Chromatin condensation was not noted, nor apoptotic bodies, and although cells became terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) positive and DNA became increasingly degraded, there was no evidence of DNA laddering. However DNA laddering was detected in senescing leaves of other species such as wheat (Caccia et al., 2001), olive (Cao et al., 2003), and five other tree species (Yen and Yang, 1998). Chromatin condensation was also reported in both tobacco and the monocot *Ornithogalum virens* (Simeonova et al., 2000). There was no clear reduction in mitochondrial membrane potentials in *Pisum sativum* mesophyll cells undergoing senescence (Simeonova et al., 2004) indicating that if mitochondria are associated with this PCD system it is not in the same way as in animal apoptosis. Chloroplast disassembly seems to be an early sign of senescence, but whether this is part of the PCD mechanism remains uncertain (Thomas et al., 2003).

Petal senescence unlike leaf senescence inevitably ends with PCD. Genomic approaches are being used to identify genes involved in petal senescence (Breeze et al., 2004) and the expression of several of the known homologs of the animal PCD-related genes, namely, Beclin, Bax inhibitor I, and dad-1 (Orzaez and Granell, 1997a, b; Wagstaff et al., 2003; Huckelhoven, 2004). Evidence from electron microscopy from *Alstroemeria* senescent petals (Wagstaff et al., 2003) clearly shows that PCD is already occurring at relatively early stages of petal senescence. In many flowers (e.g., *Arabidopsis* and tobacco), senescence, and thus PCD is triggered by ethylene (Stead and van Doorn, 1994) and sometimes associated with pollination (O'Neill and Nadau, 1997). However, in another group including lilies such as *Alstroemeria*, the role of ethylene is less Clear. Calcium signaling and GTP-binding proteins have been implicated in some species (Porat et al., 1994). Reactive oxygen species (ROS) accumulation has also been reported both in ethylene-induced (Bartoli et al., 1996) and ethylene-independent (Panavas and Rubinstein, 1998) petal senescence, together with a decrease in antioxidants (Bartoli et al., 1997). There is often evidence of tonoplast invagination (Matile and Winkenbach, 1971; Phillips and Kende, 1980) or the formation of vesicles and in the final stages only a thin layer of cytoplasm remains (Stead and van Doorn, 1994; Wagstaff et al., 2003). Changes in membrane composition, fluidity, and peroxidation occur in several species (Rubinstein, 2000). In some cases, such as carnation (*Dianthus caryophyllus*; Fobel et al., 1987), daylily (*Hemerocallis* hybrid; Panavas and Rubinstein, 1998), and rose (*Rosa* hybrid; Fukuchi-Mizutani et al., 2000), lipid peroxidation (e.g., through the action of lipoxygenases (LOX) may be one of the key factors effecting loss of membrane integrity. However, in other species like *Alstroemeria* (Leverentz et al., 2002), loss of membrane semipermeability was chronologically separated from LOX activity that had declined by over 80% by the onset of electrolyte leakage. However, loss of membrane function is the result of concerted activities of several enzymes and cannot be related to the function of a single enzyme (Paliyath and Droillard, 1992; Chapter 9). DNA laddering has been found in some petals such as *Alstroemeria* (Wagstaff et al., 2003) and gladiolus (Arora and Singh, 2006; Fig. 5.1) and pea (Orzaez and Granell, 1997b), and both nucleases (Panavas et al. 1999; Breeze

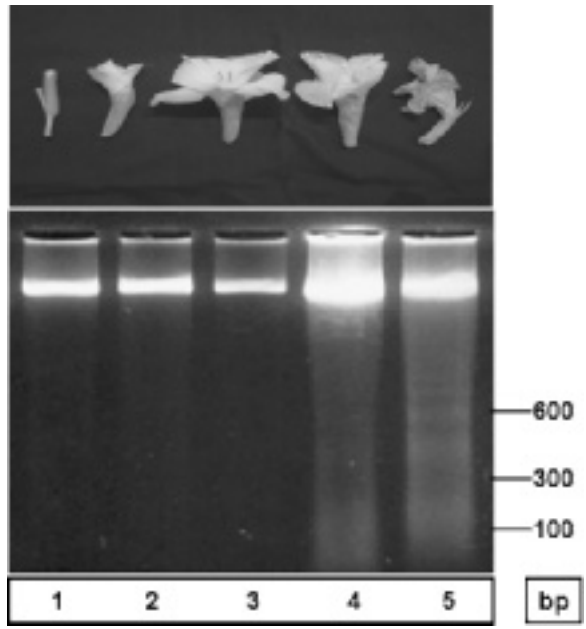

Fig. 5.1 DNA fragmentation detected in petals from various developmental stages in gladiolus (Arora and Singh, 2006).

et al., 2004; Langston et al., 2005), and proteases (Stephenson and Rubinstein, 1998; Wagstaff et al., 2002; Arora and Singh, 2004) are upregulated during petal senescence in several species. In *Sandersonia aurantiaca*, a member of a class of cysteine proteases carrying the KDEL C-terminal motif is expressed during senescence (Eason et al., 2002), which shows homology to a protease from *Ricinus communis* implicated in ricinosome-mediated endosperm PCD (Gietl et al., 1997). This suggests a mechanism for petal cell PCD, at least in this species, which may parallel the vacuole-driven autophagous model, previously described in the *Ricinus* endosperm.

5.5 Senescence signals: Cell sensitivity

The two main correlative events in plant senescence (i.e., pollination versus petal senescence and grain filling versus leaf senescence) indicate that at least in those cases signals that initiate the senescence program are produced. Strictly speaking, however, these signals just hasten or coordinate the senescence program rather than being the real event: petals eventually senesce even in the absence of pollination (O'Neill and Nadeau, 1997), and leaves eventually die even without flower or seed forming (Wilson et al., 1992).

It has been proposed that leaf senescence is triggered by age-related decline in photosynthetic processes (Hensel et al., 1993). A possible metabolic control of senescence has also been proposed (Quirino et al., 2000). The onset of senescence in the leaf has been assigned to a very early point of time when the first reduction in photochemical efficiency is detected and *cab* transcript levels begin to decline, but no visible sign of senescence and no expression of *SA*G 12 are observed (Hinderhofer and Zentgraf, 2001). As well, a careful

examination on the senescence program in rice clearly showed the asynchronous patterns of senescence among three different regions in senescing coleoptiles (Inada et al., 1998). In other systems, guard cells of the stomata and cells adjacent to the vascular tissue often remain well and alive, while the rest of the cells are becoming fully senescent (Bieleski, 1995; Sakurai et al., 1996; Willmer and Fricker, 1996). In summary, although we know that a plant cell/organ has to acquire a senescence-competence stage before it can be induced to senescence, the molecular basis for this change in cell competence remains unknown.

5.6 Cellular changes during senescence

Apoptosis is recognizable by a series of morphological and biochemical hallmarks including nuclear condensation, membrane blebbing, oligonucleosomal DNA fragmentation, and the formation of apoptotic bodies (Hacker, 2000) (Fig. 5.2). A number of noncanonical cell death processes have been reported in animal systems (Kitanaka and Kuchino, 1999), leading to the introduction of new morphological categories. Together with the standard apoptotic cell death (renamed as Type 1 physiological cell death), Type 2 (autophagic degenerative cell death) and Type 3 (nonlysosomal disintegration) categories are proposed. According to Bursch (2001), these categories should not be considered as mutually exclusive phenomena. On the contrary, they could reflect a certain flexibility of the cells to respond to different external conditions. It is probably in this flexible context of physiological cell death where the study of the morphological changes associated with plant senescence and its comparison with animal systems becomes more fruitful. The characterization of a certain morphotype (or morphotypes) associated with plant senescence should help in the identification of the molecular pathways that conform it.

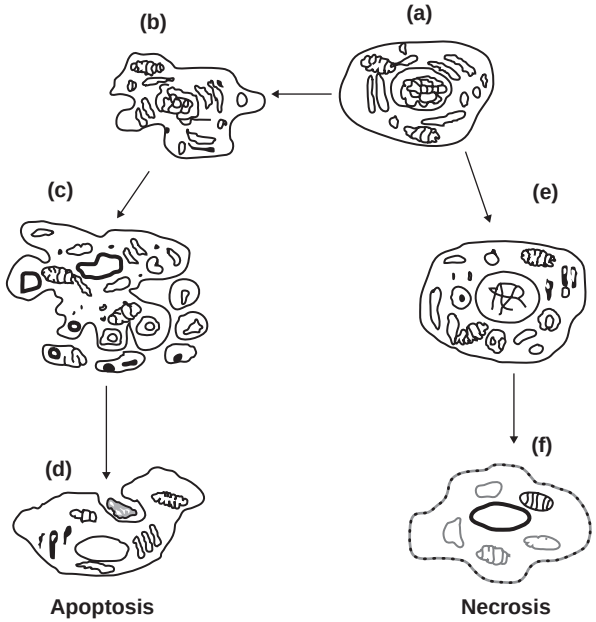

Fig. 5.2 Morphological differences between apoptosis and necrosis (Studzinski, 1999).

5.6.1 Degeneration of chloroplasts

In photosynthetic tissues, one of the first observable changes is the degeneration of chloroplasts. Usually, these organelles undergo a number of sequential changes that include, in this order, dilation and breakage of thylakoids, increase in number and size of plastoglobuli (osmiophilic globules found in electron microscopy analysis), and a decrease in pigment content (Barton, 1966; Nii et al., 1988; Simeonova et al., 2000). Inada et al. (1998) observed an early degradation of chloroplast DNA (cpDNA) prior to the degeneration of the organelle during the senescence of rice coleoptile. cpDNA appears to be degraded within the chloroplast itself, and the role of a Zn^{2+}-dependent nuclease has been suggested (Sodmergen et al., 1991). Senescent leaf plastids (gerontoplasts) are smaller than green chloroplasts, with degenerated membrane systems and stroma, and larger plastoglobuli. Surprisingly enough, gerontoplasts maintain certain integrity during early senescence and are even able to redifferentiate into chloroplasts under certain conditions (Zavaleta-Mancera et al., 1999). The number of plastids remains constant until the later stage of senescence by the time RbcS and LHC have already diminished substantially (Martinoia et al., 1983). Evidence for vacuolar autophagy of senescing chloroplast has been obtained (Minamikawa et al., 2001), and a mass exodus from senescing chloroplast has been described (Guiamet et al., 1999). These observations seem to indicate that, although the degeneration of the chloroplast functions in an autonomous manner at the early stages, probably later in the pathway, the autophagosomal mechanism is operational for chloroplast degradation.

5.6.2 Degradation of mitochondria

In contrast, mitochondria seem to maintain their integrity even in late stages of senescence with little or no degradation observed in mitochondrial DNA (Inada et al., 1998). A possible explanation is the need for ATP supply for the correct dismantling of cellular constituents. At least during the senescence of *Vigna mungo* cotyledons, the degradation of mitochondria is known to be accomplished by autophagosomes (Toyooka et al., 2001). In animal cells, the outer mitochondrial membranes act as a sensor of cellular stress, releasing apoptogenic factors (cytochrome *C*) that trigger cell death. Evidences in this direction reported in plant cells are too far to be conclusive, and refer to the PCD events taking place during stress and defense response (Balk et al., 1999; Sun et al., 1999). Observations made in petunia concluded that the release of cytochrome *C* is not a feature of PCD, at least during petal senescence (Xu and Hanson, 2000).

5.6.3 Degradation of other organelles

The nuclei of senescing cells are also subjected to modifications. Chromatin condensation is reported in relatively early stages during senescence of rice coleoptiles (Inada et al., 1998), petal senescence in carnation (Smith et al., 1992), and carpel degeneration in pea (Orzaez and Granell, 1997b). Condensation is often accompanied by the degradation of nuclear DNA, as highlighted by the TUNEL reaction (Fig. 5.3), which detects the presence of free 3′OH ends as a result of endonuclease digestion. Senescence-associated TUNEL-positive nuclei have been found in green tissues (Orzaez and Granell, 1997a; Yen and Yang, 1998; Kawai and Uchimiya, 2000), as well as in nonphotosynthetic tissues like flower petals

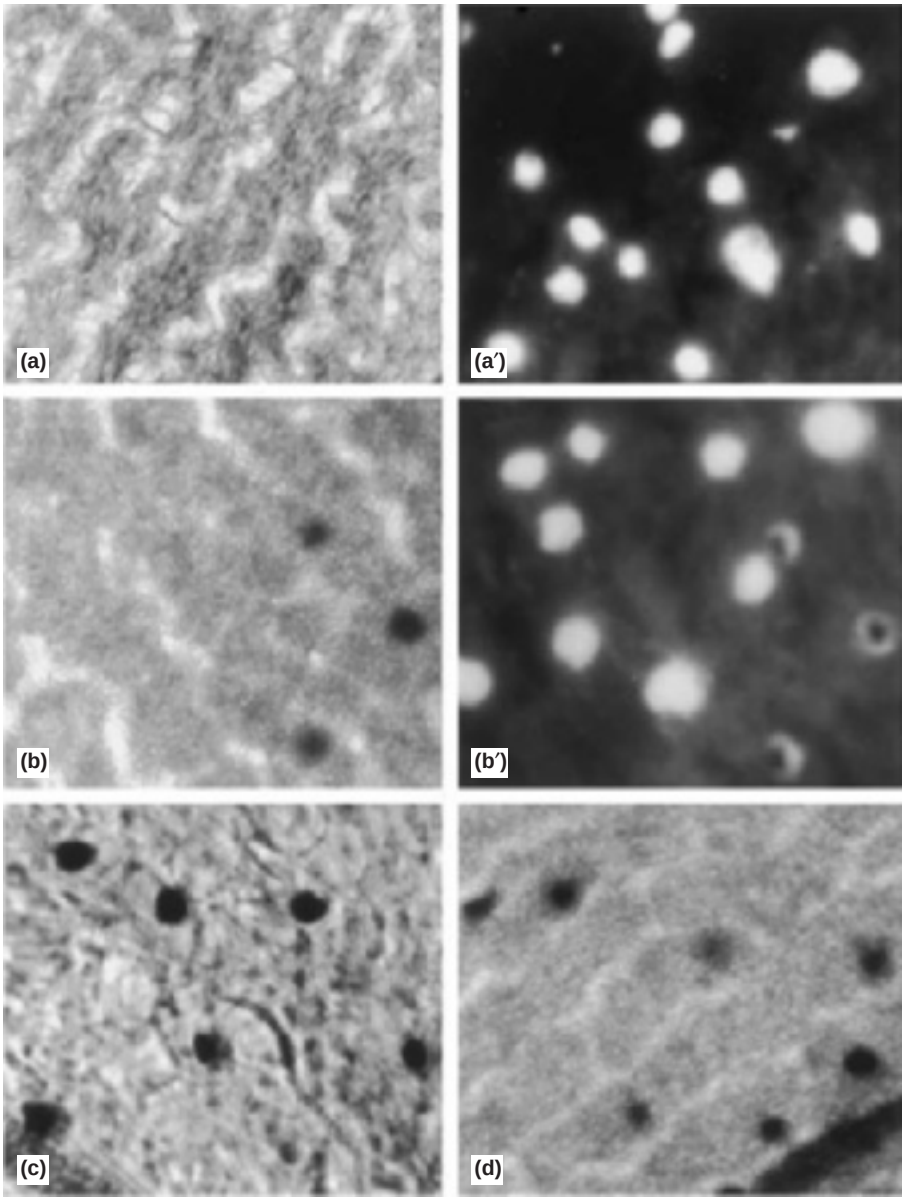

Fig. 5.3 TUNEL reaction. Whole mounts of petal pieces collected 2 days after anthesis were assayed with the TUNEL reaction. Increase in DNA ends was highlighted by addition of nucleotide analogs by using TdT, and the incorporated analogs decorated with the use of alkaline phosphatase linked to antibodies against the nucleotide analogs and NBT-BCIP substrates. (a) Petals treated with STS at stage d-1. (b–d) Nontreated petals. (a′, b′) Same as (a) and (b) but after TUNEL staining petals were incubated with Acridine Orange to visualize the nuclei (Orzaez and Granell, 1997a).

(Orzaez and Granell, 1997b; Xu and Hanson, 2000). Often, chromatin DNA degradation takes place preferably at the level of the internucleosomal space, rendering fragments of discrete size that can be visualized by electrophoresis resulting in a typical DNA ladder. Internucleosomal DNA cleavage has been considered as one of the hallmarks of apoptosis. However, its occurrence during plant senescence does not seem to be universal. Even among different organs within the same plant, the pattern of DNA degradation seems to differ. In rice, for instance, Kawai and Uchimiya (2000) reported DNA laddering during senescence in the coleoptile. In contrast, Lee and Chen (2002) found that the nuclear DNA fragmentation during rice leaf senescence is not accompanied by generation of oligonucleosomal DNA fragments. During coleoptile senescence in rice, TUNEL-positive cells are restricted to the epidermis (Kawai and Uchimiya, 2000). During the senescence of unplanted pea carpels, TUNEL labeling is restricted to well-defined areas such as the funicular-ovule joining region or the ovule external tegument. The rest of the cells in the degenerating ovule seem to dismantle its nuclear DNA without showing detectable TUNEL labeling (Orzaez and Granell, 1997a). In some senescence processes, DNA fragmentation occurs only at the very last stages (Gietl and Schmid, 2001).

Whereas the fate of the nucleus seems to differ from one form of senescence to another, virtually all the ultrastructural studies reported significant changes in the vacuolar system. At very early stages, carnation petals showed an increase in vacuolar-derived vesicles prior to other visible signs of degeneration (Smith et al., 1992). In a later stage, the central vacuole enlarges displacing nucleus and cytoplasm. Invaginations of the tonoplast into the vacuole, occasionally containing cytoplasmic material and organelles, are widely reported in different stages of senescence (Matile and Winkenbach, 1971; Inada et al., 1998).

Plant vacuoles are multifunctional organelles, and a specific type acts as lytic compartments in the degradation of cellular components (Marty, 1999). These lytic vacuoles accumulate hydrolytic enzymes analogous to the lysosomal enzymes of yeast/animal cells and are known to play a central role in macroautophagy, a major route in protein and organelle turnover, which contributes to the maintenance of cellular homeostasis. During macroautophagy, portions of the cytoplasm, including organelles, are surrounded by double-membrane structures known as autophagosomes and transported to the lytic vacuole. In plants, examples of macroautophagy have been described during nutrient starvation of suspension-cultured cells (Moriyasu and Ohsumi, 1996) and during dark-induced degradation of rubisco in chloroplasts of detached French beans (Minamikawa et al., 2001). Also, during natural endosperm senescence of germinating castor beans, glyoxysomes, mitochondria, segments of the endoplasmic reticulum (ER), and ribosomes are finally removed from the cytosol by autophagic vacuoles (Gietl and Schmid, 2001). Moreover, during the senescence of *V. mungo* cotyledons, ultrastructural analysis showed that macroautophagic processes are active in the degradation of both nutrient reserves and cellular components. Starch granules are sequestered by de novo formed membranous structures and transported to the lytic vacuole, where its degradation seems to take place. In parallel, autophagosomes-containing mitochondria and cytoplasm are also fused to the vacuole. Interestingly, while the cellular dismantling takes place both in natural conditions and in organs detached from the plant, remobilization of nutrients represented by the breakdown of polysaccharides occurs only when the organs remain attached to the plant (Bieleski, 1995; Toyooka et al., 2001). In many cases, cell compartmentalization (integrity of membranes) is maintained until very late in the program. Acidification, probably due to a change in permeability or

rupture of the tonoplast, causes the activation and exposure to the cytosol of endopeptidases stored in newly formed ER-derived vesicles (ricinosomes; Schmid et al., 1999). Once compartmentalization is lost, the contents of the lytic organelles are free to digest the remnants of cell. This represents the latest stage of senescence where homeostasis is irreversibly lost.

5.7 Molecular changes associated with plant senescence

As indicated earlier, the program of senescence requires specific transcription and translation. Over the past several years, the introduction of novel techniques for the isolation of differentially regulated genes (i.e., differential display, subtractive hybridization, etc.) has led to the isolation of many genes whose transcription levels are upregulated during senescence (John et al., 1995; Buchanan-Wollaston, 1997; Lee et al., 2001). As a result, the catalog of senescence-associated genes (*SAGs*) reported to date is hard to compile. More recently, the introduction of DNA microarrays for the profiling of gene expression is starting to provide large amounts of expression data that will help to complete the picture of genetic changes associated with plant senescence (Zhu et al., 2001; Chen et al., 2002). Together, the set of data available so far is providing clues as to the molecular constituents of senescing cells. There are two important subprograms which define molecular changes during the plant senescence:

5.7.1 The nutrient salvage subprogram

Many of the genes and activities associated with senescence can be included in this subprogram. Proteases are probably the most conspicuous group of proteins whose mRNA levels are reported to increase during plant senescence (Granell et al., 1992; Beers et al., 2000; Arora and Singh, 2004). Many of them belong to the group of cysteine endproteases (C13 class; Granell et al., 1992), which probably participate in the remobilization of N and later in the salvage program associated with senescence. Cysteine proteases are reported to be upregulated during the senescence of leaves (Drake et al., 1996), but also in reproductive organs like flowers (Wagstaff et al., 2002; Arora and Singh, 2004) and other senescent organs such as unplanted ovaries (Granell et al., 1992; Cercos et al., 1999). Because most of them contain vacuolar-targeting signals, they are likely to participate in the digestion of proteins delivered by the autophagosome for N/C retrieval or in general digestion once the tonoplast has been lost at the later stages of senescence. Most of these proteases are synthesized as proenzymes and sorted to vacuole where they become activated.

A different pathway is followed by a group of cysteine endo peptidases with a C-terminal KDEL sequence for retention in the ER, as the CysEP protease from castor bean-senescing endosperm (Schmid et al., 1999). Orthologs of CysEP are also found in senescing daylily petals (Valpuesta et al., 1995), cotyledons of mungbean (*V. mungo*) and vetch (*Vicia sativa*), and seed pods of maturing French bean (*Phaseolus vulgaris*) (Schmid et al., 1999). CysEP is accumulated as a precursor in specialized organelles, the ricinosomes, which are originated as budding vesicles arising from the ER. With the progress of senescence, ricinosomes disintegrate, liberating the active form of CysEP into the cytoplasm (Schmid et al., 1999). Although the substrate specificity of CysEP is unknown, its containment in specialized vesicles and subsequent liberation during senescence seems indicative of a highly regulated

process for the release of proteolytic activities, which resembles the regulation of PCD during apoptosis in animal cells (Gietl and Schmid, 2001).

Also in the extracellular matrix, a metalloproteinase (CSL-MMP) induced late in the senescence process of *Cucumis* cotyledons (along with DNA laddering) suggests that these protease activities may help to eliminate the later cell remnants, as has been described for its orthodoxy in animals (Delorme et al., 2000).

Lately, many of the components of the autophagy molecular machinery in *Arabidopsis* have been identified based on their homology to the genes identified in *Saccharomyces cerevisiae* (Hanaoka et al., 2002), which makes possible to study the contribution of this pathway to senescence. Recent evidence indicates that the APG pathway is upregulated during senescence, although compared to SEN1 mRNA (a vacuolar cysteine endopeptidase), accumulation of *APG7* and *APG8* mRNAs occurs rather late, suggesting that the APG system may function later than other molecular responses induced during senescence (Doelling et al., 2002). Disruption of either of two *APG* genes (*APG9* or *APG7*) in *Arabidopsis* produces an accelerated senescence phenotype (both natural and dark-induced) and starvation-induced chlorosis. This indicates that the APG pathway is required for efficient nutrient recycling and senescence in *Arabidopsis* (Doelling et al., 2002; Hanaoka et al., 2002).

It is tempting to speculate that, in addition to their role in N-mobilization, some plant proteolysis activities (Delorme et al., 2000) could be involved in a cascade of proteolysis activation regulating PCD event. Elucidation of the substrates for these activities is needed to clarify their role during senescence.

In addition to substrate and proteases being differently compartmentalized and only activated and released at the right time, substrate susceptibility for proteolysis appears to be important in senescence and in other stress-related processes (Pefiarrubia and Moreno, 1990; Cotelle et al., 2000). This is best exemplified for chlorophyll a/b binding proteins (Cab proteins) that remain stable as far as they are complexes with chlorophyll. Removal of chlorophyll during senescence increases dramatically Cab susceptibility to proteolysis. Inhibition of chlorophyll degradation, as in *stay-green* mutants, increases notably the stability of Cab, while the rest of the senescence proteolysis and other senescence processes continue as normal (Thomas and Howarth, 2000). Not only structural changes in substrate susceptibility are important for proteolysis: binding of 14-3-3s proteins to a wide range of plant proteins can also induce changes in their stability (Cotelle et al., 2000). The extent to which this mechanism is important for senescence is indicated by the *stay-green* phenotype and delayed leaf senescence of transgenic potatoes overexpressing 14-3-3s proteins (Markiewicz et al., 1996). This suggests that proteolysis of the 14-3-3s targets participates in the progression of senescence. The regulation of 14-3-3s levels could function as a mechanism to modulate senescence, probably through the protection exerted by 14-3-3s proteins on their targets. Conversely, transgenic plants with diminished levels of 14-3-3s proteins showed an early senescence phenotype (Wilczynski et al., 1998), thus closing the circle and further supporting the relevance this mechanism may have during senescence.

The nature of SAGs-encoding activities, which are involved in nutrient salvage program, ranges from glyoxysomal enzymes involved in fatty acid mobilization, like 3-ketoacyl-CoA thiolase in pumpkin (Kato et al., 1996) or malate synthase gene in cucumber (Graham et al., 1992; Buchanan-Wollaston, 1997) to cell wall–metabolizing enzymes (Lee et al., 2001) such as β-glucosidases (Callard et al., 1996). Nucleases probably involved in phosphate

mobilization are also found associated with senescence (Perez-Amador et al., 2000; Lers et al., 2001). Interestingly, the tomato ribonuclease LX contains an HDEL signal for ER retention (Lehmann et al., 2001). LX is expressed not only during leaf senescence but also during germination and xylem differentiation. It is assumed that RNase LX accumulates in an ER-derived compartment and is released by membrane disruption into the cytoplasm of those cells that are intended to undergo autolysis, in a similar fashion as reported in the case of castor bean KDEL-cysteine protease described earlier. Also, SAG-encoding enzymes for glycolysis/gluconeogenesis are identified, suggesting the activation of alternative pathways for obtaining energy to sustain the senescence program (Buchanan-Wollaston, 1997; Buckner et al., 1998).

A large number of genes are known to be downregulated during senescence. Some of them refer to the photosynthesis-associated genes, but others such as AGL15 (Fernandez et al., 2000) may have a regulatory role and, therefore, can be important in the modulation or establishment of the program.

5.7.2 Cell preservation subprogram—protection against oxidative stress

Senescent cells are especially sensitive to different stresses (Arora et al., 2002). Protection of the senescent cell against oxidative damage is exerted at many levels: induction of protective antioxidative activities, elimination of photooxidative molecules, production of sunscreens, etc. All of these are upregulated during senescence. One of the reasons for this appears to be the high phototoxicity (ROS generated) of unbound chlorophyll and its products (Mach et al., 2001). In the presence of light and oxygen, the unbound chlorophyll released from the membrane during senescence would produce singlet oxygen and cause photooxidative damage thus jeopardizing nutrient retrieval from these cells. To prevent this, chlorophyll is not metabolized and mobilized as nutrients, but is stored in the vacuole as any other xenophobic substance (Hortensteiner and Feller, 2002). Consistent with this, gene expression profiles during senescence overlap with signaling pathways related to stress (Buchanan-Wollaston, 1997; Rubinstein, 2000). Examples of stress-related *SAGs* include glyoxalase II gene from *Arabidopsis* (Quirino et al., 1999), together with a variety of genes involved in oxidative stress like Fe(II) ascorbate oxidase in *Arabidopsis* (Callard et al., 1996), catalase in *Brassica* (Buchanan-Wollaston and Ainsworth, 1997), among others. The working hypothesis is that antioxidant activities are elevated to keep the cell in a viable stage for as long as the nutrient mobilization mechanism is in place. The chloroplastic form of glutamine synthase from pea leaves is degraded more rapidly than the rest of the chloroplast enzymes involved in carbon assimilation (Thoenen and Feller, 1998). In contrast, the cytoplasmic form remains stable and localized in the vascular elements of leaves (Sakurai et al., 1996) whose cells show a much delayed senescence to help in the remobilization of nutrients.

5.7.3 Transcriptional activation cascade in senescence PCD

Early searches for genes induced during senescence failed to identify transcription factors associated with senescence. This, together with the lack of detection of obvious common upstream sequence homologies between different *SAGs* identified at that time (Buchanan-Wollaston, 1997; Gan and Amasino, 1997) casts some doubts on the existence of a real

(transcriptional activation) program for senescence (Bleecker, 1998). A number of potential transcription factors are now known to be associated with senescence (Hinderhofer and Zentgraf, 2001; Robatzek and Somssich, 2001; Yang et al., 2001; Zentgraf and Kolb, 2002). Furthermore, a comprehensive analysis of the promoter region from 23 genes that are induced during leaf senescence indicates that the consensus W region for binding of WRKY proteins is highly enriched in those genes (Zhu et al., 2001).

A recombinant form of one of those factors, WRKY53, is able to bind to W boxes present in the promoters of a number of genes, some of which happen to be transcriptional factors themselves (like other WRKY members), or genes whose mutation alters the senescence program (like the F-box protein ORE9) or other *SAGs* (like *SAG12*,*CATs*, etc.). This suggests the possibility (a) that WRKY53 is part of a transcriptional activation cascade and (b) that some of the SAGs are to be the targets of WRKY53, and therefore responsible for its activation during senescence. Interestingly, both age of the leaf and age of the plant drive the same set of *SAG* genes including a set of transcription factors (Hinderhofer and Zentgraf, 2001).

Another study identified a number of transcription factors that are shared between senescence and pathogen defense programs. Indeed, the WRKY genes are upregulated during pathogen infection, wounding, and senescence, and some of them are shared between different programs (Eulgem et al., 2000; Chen et al., 2002). More specifically, AtWRKY4, 6, and 7 are highly induced during senescence. Transcription factors induced during senescence are not restricted to the WRKY but include other families such as AP2/ErebpS, Myb, bZIPs, and homeobox (Chen et al., 2002).

The complexity of the transcriptional activation network can be illustrated with the candidate *cis*-acting elements present in the promoter in any one of the senescence-inducible WRKYs. WRKY6 promoter contains tissue-specific motives, W boxes that have been found to negatively influence the expression of *AtPRl* gene, an *asl-like* element and MYB recognition region that have been shown to bind tga factors. These interactions between *mis*-acting elements and *trans*-acting factors are likely to mediate *AtWRKY6* gene expression in response to SA, JA, and auxin (Robatzek and Somssich, 2001).

When AGLI5, a member of the plant MADS domain family of regulatory factors, was expressed in *Arabidopsis* under the control of a 35S promoter or under glucocorticoid-inducible promoter, an important increase in the longevity of both sepals and petals was observed (Fernandez et al., 2000; Fang and Fernandez, 2002). AGL15 is normally expressed in juvenile tissues. Moreover, it was found that overexpression only affects longevity if the transgenic is switched on around the time of flower opening (before senescence starts). Together, these observations suggest that AGL15 contributes to maintain the juvenile non-senescent stage probably by repressing the senescence program (Fang and Fernandez, 2002). Similarly, transgenic tobacco plants expressing the maize homeo box gene *knotted1* under the control of the senescence-activated promoter SAG12 resulted in increased cytokinin content and delayed senescence (Ori et al., 1999). It is proposed that ectopic expression of *Kn1*, a gene whose function in wt meristems seems to maintain a population of indeterminate cells in which differentiation is delayed or inhibited, would block developmental progression to senescence in fully mature leaves of *SAG12::Kn1* plants. These results reveal the existence of both positive and negative transcription signaling to the developmental senescence program, and that probably the establishment of the senescence program may require first switching-off juvenile factors such as AGL15 or *Kn1*.

5.7.4 Elements of the upstream senescence-signaling PCD pathway

Receptor-like protein kinases have been implicated in senescence signaling (Hajouj et al., 2000; Robatzek and Somssich, 2002; Arora et al., 2006). It is known that receptor-like kinase serves as receivers and transducers of external and internal stimuli, acting through phosphorylation/dephosphorylation cascades that eventually lead to changes in gene expression. The senescence-associated kinase receptor gene (SARK) behaves as a typical *SAG*, which is induced by senescence-inducing factors (ethylene; jasmonate) and repressed by senescence-delaying factors (cytokinin, light). Both transcript and protein appear prior to the onset of senescence (Hajouj et al., 2000). Another receptor kinase, senescence-induced receptor kinase (SIRK), was identified by Robatzek and Somssich (2002) as one of the candidate targets of the senescence transcription factor AtWRKY6. This transcription factor is able to respond to senescence and other external stimuli often associated with senescence and plant defense (Robatzek and Somssich, 2001). As this factor does not require de novo synthesis for its activation, it can be considered an early-type element and together with other WRKY, they are likely substrate for kinase or phosphatases (Eulgem et al., 2000). In contrast to SARK that is expressed in roots and during senescence, SIRK is the only identified plant receptor kinase developmentally expressed solely during senescence. In addition to senescence, SIRK and WRKY6 participate in pathogen defense pathway. A dual function for SIRK/WRKY6 has been proposed. On the one hand, senescence would be initiated by the binding of a senescence-triggered signal to SIRK, which would lead to the expression of WRKY6. On the other hand, SIRK would activate a kinase cascade that would modify WRKY6 protein, resulting in the induction of several genes, including SIRK.

MAP kinase cascade seems to be part of the signal transduction pathway linking the senescence developmental signal and the downstream elements. Thus, MAPKK in tomato and MAPK in maize are both identified by the increase in mRNA levels and by activity gels during senescence. Interestingly, neither PGRs (like ethylene, cytokinin, GA, ABA) nor nutrient starvation has affected either the kinase activity or the transcript levels of this natural senescence-inducible MAPK (Berberich et al., 1999). The importance of MAP kinase in the transcriptional activation cascade is consistent with the constitutive expression of a set of potential WRKY effector genes observed in the MAP kinase mutant of *Arabidopsis mpk4* (Petersen et al., 2002).

Other posttranslational modifications seem to be involved in the regulation of senescence. The finding that the delayed senescence mutation *ORE9* encodes a mutated form of an F-box protein suggested that ubiquitin tagging and proteolysis of a repressor could be an upstream regulatory component of the PCD/senescence pathways (Woo et al., 2001). ORE9 is able to physically interact with ASKI (an *Arabidopsis* Skyline protein), which is a component of the SCF type of E3 ubiquitin ligase complex. As F-box proteins appear to be responsible for target specificity through protein–protein interaction motifs such as WD-40 (Callis and Vierstra, 2000); elucidation of the proteins interacting with ORE9 will be very important in understanding which factors are targeted for degradation that are important for senescence. Furthermore, this proteolytic pathway seems to be activated during senescence: the E2-ubiquitin carrier protein shows increased expression during leaf senescence (*Nicotiana sylvestris*; Genschik et al., 1994). Increased expression of a polyubiquitin gene, SEN3, has also been detected in senescent leaves of *Arabidopsis* (Park et al., 1998).

Further support for the involvement of the ubiquitin/proteasome pathway during senescence comes from the recent report that a mutant defective in *AtATE1* gene, an arginyl-tRNA:protein arginyltransferase involved in the N-end rule pathway, shows a delayed senescence phenotype in both cotyledons and rosette leaves of *Arabidopsis* (Yoshida et al., 2002a). It is possible that a cytosolic or nuclear component is repressing the progress of senescence, and this factor is destabilized during senescence by the R-transferase activity of *AtATE1* gene product. Ubiquitinated proteins are not necessarily degraded by the 26S proteasome: many yeast cell surface receptors use ubiquitination for internalization and degradation by the vacuolar proteases (Wilkinson, 1999). Whatever the case, this indicates that the N-end rule has an important role in the progression of leaf senescence.

Changes in membrane potential and internal concentrations of Ca^{2+} are part of the signal transduction pathway in animal PCD, and there are indications that this can also be the case for plants. The recent identification of a cyclic nucleotide-gated ion channel from *Arabidopsis* (*AtCNGC2*) that shows developmental regulation during the early stages of senescence in different organs, but not in late stages, indicates that this ion channel may participate in the signaling of the senescence process (Kohler et al., 2001). Furthermore, the realization that the *Arabidopsis* mutant *dnd1*, which shows reduced ability to undergo cell death when exposed to avirulent *Pseudomonas syringae*, is caused by a mutation in this same gene *AtCNGC2* (Clough et al., 2000) supports its role as a mediator in different forms of PCD.

Identification of additional regulatory elements controlling senescence in plants may come from genetic approaches. The identification of several *ore* (from Oresara, long-lived in Korean language; Oh et al., 1997) mutants indicates that the search can still be fruitful. *Ore* mutants show just delayed senescence and not a complete block in senescence. The reason for this could be multiple pathways acting in parallel to induce senescence, making genetic screens difficult or alternatively that all the alleles identified so far are weak alleles. Ore 2 and 3 came out to be alleles of the ethylene-insensitive mutant *ein2* and are therefore affected in the timing of senescence via the ethylene pathway. The nature of *old* 1, 2, and 3 (onset of leaf death) mutants of *Arabidopsis* is not known, but mutations in these genes confer an early onset of senescence (Jing et al., 2002). Characterization of the molecular basis for several senescence mutants from maize, soybean, and other plants will further contribute to our understanding of senescence (Buckner et al., 1998).

5.8 Early signal of senescence pathway

A senescence-associated decrease in membrane fluidity has been detected in all senescing tissue (Paliyath and Droillard, 1992). Changes in membrane composition occur well before the appearance of any visible symptoms of senescence in petals. There is a senescence-associated decline in the phospholipid content due to a decrease in synthesis and an increase in degradation. The ratio of sterol/phospholipid may increase as much as 2–6 times, and this leads to a decrease in membrane fluidity, which can be detected by fluorescence polarization and ESR techniques, preceding any visible symptom of senescence in petals. Changes in fluidity appear to affect the activity of several membrane-bound proteins such as ATPase and probably many other activities (Paliyath and Droillard, 1992).

In contrast to animal apoptosis, no specific protease has yet been clearly found to be involved in the initial events of senescence/PCD. A specific role for a matrix

metalloproteinase (At2-MMP) has been proposed (Golldack et al., 2002) based on the fast degradation of chlorophyll and early senescence phenotype of an *Arabidopsis* mutant in which this gene is interrupted by a T-DNA. According to these workers, senescence "sensor" is either continuously degraded or specifically activated by MMP; MMP-deficient tissues would be hypersensitized for senescence (Golldack et al., 2002). A role of At2-MMP in releasing signal molecules that trigger cell death both in senescence and in other plant PCD processes such as tracheary element (TE) differentiation (Groover and Jones, 1999; Delorme et al., 2000) has also been proposed.

5.9 Signaling cascades network

The expression of many senescence-regulated genes is also affected in other signaling pathways, indicating a crosstalk between senescence/PCD and other routes: response to environmental stress, hormones, N/C status, sugars, and amino acids, etc. In some cases, this just indicates that different endogenous/environmental signals can activate the senescence pathways. But several evidences indicate that there could be at least partially parallel signaling cascades leading to senescence.

An illustration of how the molecular mechanisms underlying senescence is part of a network of interactions in which both internal and external factors participate has been presented by He et al. (2001) using a large-scale enhancer trap strategy and expression analysis in *Arabidopsis.* Using this approach, He and coworkers identified 147 lines that showed increased expression in senescent leaves but not in nonsenescent ones, suggesting that the construct has landed in a senescence-inducible gene. Expression analysis of these genes show that 63 of them were specific for senescing leaves, but in 62 lines the reporter gene was also expressed in senescing flowers, siliques, and stems. And in a few lines (4), there was expression in young tissues. These results indicated that there must be both common and specific components among the senescence programs operating in different organs. The effect of different endogenous and environmental factors on the expression of these enhancer lines was also studied, and it gives support to the idea that each of these factors (ABA, JA, darkness, ethylene, brassinosteroids, dehydration, age, and others) contributes to the senescence program by inducing a subset of senescence-associated genes. A few of the lines showed increased expression by two or more of the factors, and preliminary attempts to construct the senescence regulatory network were presented (He et al., 2001).

One consequence of the existence of a complicated network as anticipated by Gan and Amasino (1997) is the plasticity of the program, that is, the senescence program can always proceed through other "branches" of the network. Consistently, most of the homozygous Sel knockouts showed no phenotype, and only a few displayed delayed senescence (He et al., 2001). Most interestingly, a majority (2/3) of the 43 transcription factors showing transcriptional activation during senescence (Chen et al., 2002) are also induced by stress treatments (bacteria, viruses, fungi or cold, high salt, or osmotic depending on the specific factor). Indeed, pathogens and ethylene are known to induce senescence, and similar genes have been identified with both stresses.

In other studies, some members of the bZIP gene family of transcription factors that are characterized by its induction on exposure of the plants to low temperatures increased during aging/senescence of leaves (Berberich et al., 1999; Yang et al., 2001). Interestingly, *tbzF*

transcripts that were strongly upregulated by 4°C exposure are also induced by ethylene, IAA, and JA but not by BA, and showed a typical SAG pattern during senescence.

Some elements of the signal transduction pathway upstream the transcription factors can also be shared between different stresses and senescence. Thus, the senescence-associated ZmMAPK5 is also induced during recovery after low-temperature stress (Berberich et al., 1999).

5.10 Salicylic acid

Plants possess an immune system to defend themselves against pathogen infection. An intensively studied inducible immune response occurs when a pathogen carrying an avirulence (*avr*) gene is recognized directly or indirectly by a cognate resistance (*R*) gene in the plant. This leads to activation of defenses that restrict pathogen growth in infected tissues and in noninfected tissues by a process referred to as systemic acquired resistance (SAR). These defense responses are typically accompanied by localized PCD around the site of infection in the hypersensitive response (HR; Nimchuk et al., 2003). In the absence of an *R-avr* interaction, basal resistance responses are also activated, although they may not successfully restrict pathogen growth, and disease symptoms may develop (Glazebrook et al., 1997).

The importance of salicylic acid (SA) in the induction of such resistance responses is supported by both gain- and loss-of-function evidence. SA levels increase on many avirulent and some virulent infections (Malamy et al., 1990; Métraux et al., 1990; Heck et al., 2003), and application of exogenous SA, or generation of high endogenous SA levels by expression of bacterial SA-synthesizing enzymes, is sufficient to induce resistance to many normally virulent pathogens (White, 1979; Ward et al., 1991; Verberne et al., 2000; Mauch et al., 2001). Loss-of-function analyses have relied on SA depletion by transgenic expression of a bacterial SA hydroxylase encoded by *nahG*. NahG abrogates local *R* function elicited by a range of bacterial, oomycete, and viral pathogens (Delaney et al., 1994; Rairdan and Delaney, 2002) as well as SAR (Gaffney et al., 1993) and basal resistance responses to virulent bacteria, fungi, and oomycetes (Delaney et al., 1994; Reuber et al., 1998). The involvement of SA in activation of PCD in the HR is supported by similar lines of evidence but remains less clear. SA does not induce HR-like PCD on its own in whole plants, although it may induce PCD in cell culture (Kawai-Yamada et al., 2004). HR induced by two *Peronospora parasitica* isolates avirulent on *Arabidopsis* appears to depend on SA, since *nahG* blocked the HR in response to infection, although trailing necrosis surrounding growing hyphae was still observed (Nawrath and Métraux, 1999). Similarly, *nahG* delays the HR of tobacco (*Nicotiana tabacum*) in response to tobacco mosaic virus (Mur et al., 1997). Consistent with these observations of PCD attenuation by SA removal, exogenous SA strongly accelerated HR cell death in soybean (*Glycine max*) suspension cells (Shirasu et al., 1997) and induced cell death in *Arabidopsis lsd1* mutants and *RPW8*-enhanced transcription lines kept under conditions nonpermissive for spontaneous HR-like cell death development (Dietrich et al., 1994; Xiao et al., 2003). Clearer evidence pointing to a role of SA in PCD comes from the analysis of *Arabidopsis acd* and *lsd* mutants that spontaneously activate PCD and defense responses. In many of these mutants, including *acd6-1*, *acd11*, *ssi1*, and *lsd6*, *nahG* expression completely suppresses PCD development, while this can be restored by application of SA agonists such as 2,6-dichloroisonicotinic acid (INA) and

benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH; Weymann et al., 1995; Rate et al., 1999; Shah et al., 1999; Brodersen et al., 2005). Similarly, *nahG* inhibits PCD induction by the mycotoxin fumonisin B1 (Asai et al., 2000). Thus, the clearest links of SA to PCD induction are based on analysis of NahG plants.

5.11 Regulation of senescence by sugar signaling

Coordination of development with the availability of nutrients, such as soluble sugars, may help ensure an adequate supply of building materials and energy with which to carry out specific developmental programs. For example, in vivo and in vitro experiments suggest that increasing sugar levels delay seed germination and stimulate the induction of flowering and senescence in at least some plant species. Higher sugar concentrations can also increase the number of tubers formed by potatoes and can stimulate the formation of adventitious roots by *Arabidopsis*. New insights into the mechanisms by which sugar response pathways interact with other response pathways have been provided by microarray experiments, examining sugar-regulated gene expression under different light and nitrogen conditions (Gibson, 2005).

Senescent cells show a decline in photosynthetic rate; furthermore, certain components of the photosynthetic apparatus are the early target for proteolysis, and the mRNA transcripts for such proteins are downregulated during senescence. Increasing evidence supports that sugars, the primary products of photosynthetic activity have, in addition to their essential roles as substrates and source of energy, important functions in signaling (Rolland et al., 2002).

The evidence regarding this puzzle is fragmentary and sometimes contradictory. Thus, sugars are known to repress photosynthetic gene expression (occurring during senescence). Contents of glucose and fructose in leaves increased with age, while starch content diminished (Wingler et al., 1998). Moreover, *glucose-oversensitive* mutants of *Arabidopsis* such as *hysl/cpr5* are indeed selected by the hypersenescence phenotype (Yoshida et al., 2002b). Senescent petals also contain enough levels of reducing sugars, and therefore limited respiratory substrate is unlikely to be a major factor in petal senescence. Sucrose itself may accumulate at high levels in senescing tissues (Crafts-Brandner et al., 1984), although in this case sucrose is likely to be synthesized via glyoxylate pathway.

How can sugars act as signals for senescence? Often, a key role on sage signaling has been attributed to hexokinase (HXK). HXK is known to be a glucose sensor that monitors sugar levels and responds by modulating gene expression and multiple plant hormone-signaling pathways. In addition, the expression of HXK correlates well with the rates of leaf senescence. This could indicate that the plant coordinates the activity sources and sink organs (carbohydrate importing or exporting sites) according to the internal and external conditions (Sheen et al., 1999; Smeekens, 2000). Furthermore, the transport of monosaccharides appears to increase during advanced leaf senescence as shown for the homolog SFPI (Quirino et al., 2001).

Like other terminally differentiated organs, mature flower petals and leaves contain highly active invertases, whose activities decrease during senescence and as a consequence, the ratio of sucrose to reducing sugars increases. It has been proposed that de novo synthesis of an invertase inhibitor is the reason for the decrease in invertase activity. This will leave more sucrose available for mobilization to other parts of the plant.

Most interesting is the interaction between cytokinins and sugars and its relation with senescence. Cytokinins have been found to increase extracellular invertase and sugar uptake in different plant systems. One hypothesis is that this would lead to a delay in senescence by increasing the sink strength of the organ. Indeed, contrary to what happens in wild-type plants, transgenic *sag12::ipt* plants show an increase in invertases with the age of the leaf that accompanies the delay of senescence. Furthermore, *sag12::cin1* plants that express invertases under the control of a senescence-inducible promoter show a delay in senescence. And finally, transgenic plants carrying a cytokinin-inducible promoter *cin6* linked to an invertase inhibitor gene show no delay in senescence. All these experiments give further support to the importance of achieving high levels of invertase/sugars for a delayed senescence. This is however far from being the complete history as high levels of sugars are also inducers of senescence and, indeed, some of the *sag12::cin1* lines show a premature senescence rather than a delay, indicating that an optimum range of sugar levels is important and that a fine control of senescence by sugar levels is in action.

Although analysis of promoters of different photosynthesis-related genes that showed decreased expression during senescence did not reveal common regulatory elements for sugar regulation (Sheen et al., 1999), specific regulatory elements have been detected in the promoters of α-amylase, malate synthase, and *RbcS* genes that are required for sugar repression. Interestingly, SPF1, a transcription activator that binds SP8 motif of sugar-regulated genes contains orthologs in *Arabidopsis* that belong to the WRKY family of transcription factors. Interestingly, some of these factors, which are known to bind W boxes found in promoters of α-amylase and defense-related genes (Du and Chen, 2000), are also induced during senescence.

The sugar connection can be at the crossroads of the multiple environmental and endogenous factors that affect senescence. This can be accomplished by the existence of regulatory sequences present in the promoter regions. Thus, regulatory sequences present in sugar-regulated genes include the G-box (Martinez-Garcia et al., 2000) and the related ABA responsive elements (Pla et al., 1993), which could channel the input signals from the environment through phytochrome/ABA signals (light/developmental or environmental stress). Alternatively, the interaction can be at the level of elements of the signal transduction pathway of senescence or any of the hormones that affect senescence. In this same direction, the ethylene signal transduction element EIN2 contains a cytoplasmic carboxyl terminus that is sufficient to activate downstream elements in the ethylene pathway and has structural homology to the yeast glucose sensor Snf3 (Alonso et al., 1999). It is thus possible that the ethylene pathway may integrate signals from sugar levels and senescence. The existence of a network is highlighted by the several sugar-insensitive *Arabidopsis* mutants (insensitive or uncoupled), which are affected in hormone action (Gazzarrini and McCourt, 2001). Unfortunately, most of what is known on the interaction of sugars and hormones has been studied in *Arabidopsis* during early seedling growth and not in senescent organs. It is tempting to speculate that the hypothetical model depicted for early seedling growth in *Arabidopsis* can be extended to include senescent leaves (Gazzarrini and McCourt, 2001). In this view, sugars obtained through hydrolysis of storage compounds from senescent leaves (lipids, starch, fractions, etc.) would signal an increase in the levels of ABA. Contrary to the effect on germinating seedlings, where sugar signaling causes growth arrest, the senescent leaf will inhibit photosynthesis and activate the lipid/starch/fraction breakdown pathway.

5.12 PCD during petal senescence

Recently, there has been much controversy over the use of the terms "senescence" and "programmed cell death" (PCD), especially with regard to leaves (Thomas et al., 2003; van Doorn and Woltering, 2004). In flowers it seems that the distinction is largely unnecessary. The deterioration of a flower is certainly programmed, and is not a reversible process and inevitably leads to flower senescence. Thus, the terms used essentially interchangeably, using PCD more often when discussing the death of individual cell types, and senescence for whole organs.

Selective removal of reproductive structures is not unique to plants. However, unlike in mammals, both male and female reproductive structures in plants are only retained while they are needed, and are developed de novo in perennial species during the following reproductive cycle. The longevity of the flower is species specific and carefully tailored to its ecological requirements. This is important because the flower can be a substantial sink on the plant's resources, and as such, is energetically expensive to maintain beyond its useful life (Ashman and Schoen, 1994). Another important reason for floral death after pollination is to remove it from the population so that it does not compete for pollinators with the remaining blooms. One of the key triggers for petal death is pollination, which initiates a series of physiological events, orchestrated by plant growth regulators (PGRs). Ethylene is a clear regulator of petal senescence in some species (Stead and van Doorn, 1994); however, in other species including lilies such as *Hemerocallis* (daylilies), *Gladiolus*, and *Alstroemeria*, it appears to play little or no part (Woltering and van Doorn, 1988; Wagstaff et al., 2005; Arora et al., 2006). How petal senescence in these species is triggered and orchestrated remains unknown. Given the failure to find a common regulator for these species and their taxonomic diversity, it seems likely that several interrelated mechanisms may be at play. Resource allocation has been one trigger proposed, and indeed removal of lower flowers in a cyme can lead to increased longevity of the first flower (Chanasut et al., 2003). However, this is clearly not a full explanation for all ethylene-insensitive species. An important feature of floral death is that the different floral organs play very different roles. Hence, their lifespan needs to be appropriately coordinated. Likewise, the purpose and fate of the dying cells depends on the organ and tissues involved. At a whole organ level, petals, anthers, and stigma are no longer required following pollination, whereas the ovary will mature to contain the developing seeds. In many species, there is also a mechanism for rescuing resources from the degenerating organs such as petals, and diverting them to other parts of the plant such as the developing ovary (Stead and van Doorn, 1994). At a tissue and cell level, the situation is even more complex as there is a requirement for some reproductive tissues and cells to die to ensure correct development. For example, the tapetum must degenerate for pollen to develop properly, and synergid cells must die to allow fertilization. However, the fate of the dead cells is very different. In the case of the tapetum, cell contents are used to form the coat of the pollen grains, whereas removal of synergid cells is required for fertilization to occur (Christensen et al., 2002). Some types of cell death in floral organs also depend on specific genetic interactions. PCD occurs as a result of incompatible pollination events (Thomas and Franklin-Tong, 2004) and also as a result of defects in pollen development displayed in cytoplasmic male sterile lines (Balk and Leaver, 2001). Thus, important questions with regard to cell death in reproductive organs are the following: How do the cells perceive and respond to death signals, or how do they know when to die? Are the primary signals

processed in the same way by the different organs and cells? Is the type of PCD in floral organs also found in other plant tissues and organs?

5.13 Death signal for cell

In some species, pollination dramatically shortens floral lifespan. For example, orchid flowers will last several months but senesce rapidly once pollinated. In several species, including *Petunia*, tobacco, carnation, and orchids, senescence is mediated by the evolution of ethylene following contact between pollen and the stigma surface, which precedes fertilization (O'Neill, 1997). However, the exact nature of the primary signal resulting in ethylene evolution has not been established, although other PGRs and low-molecular-weight compounds have been implicated (O'Neill, 1997). In carnations, ethylene produced from the pollinated stigma is translocated, via the style and ovary, to the petals. Here, it upregulates ethylene biosynthetic genes and induces the production of ethylene in the petals (ten Have and Woltering, 1997). Once initiated, the evolution of ethylene becomes autocatalytic (Woodson and Lawton, 1988). This strongly suggests that promoters of the ethylene biosynthetic genes respond to ethylene and contain ethylene-responsive elements (EREs). To date, this has not been verified, although an ERE from a senescence and ethylene-regulated gene in carnation bears similarities to the ERE from an ethylene-responsive fruit-ripening gene, E4, suggesting commonality of transcription factors in these two processes (Deikman, 1997). The response to ethylene is regulated by the production of ethylene receptors, but how this regulation is achieved is not clear. In tomato an ETR1 ("ethylene-resistant")-type ethylene receptor was not transcribed in young flowers or senescent flowers, but only in mature flowers (Payton et al., 1996). Furthermore, ethylene receptor expression may itself be regulated by ethylene production. In pea, transcripts of an ERS ("ethylene response sensor")-type ethylene receptor were reduced when unpollinated flowers were treated with an inhibitor of ethylene biosynthesis (Orzaez et al., 1999). So the balance between receptor production and ethylene sensitivity is clearly regulated at several levels. Notably, in species in which ethylene is a major regulator, ethylene-independent signals are also present. Disruption of ethylene signaling or biosynthesis in carnation and petunia results in delayed floral death, but the flowers do eventually die (Michael et al., 1993). Perhaps it is these endogenous signals that are active in species where ethylene is not a major regulator. Several global transcriptome studies (e.g., *Alstroemeria*, Breeze et al., 2004; *Iris*, van Doorn et al., 2003) have attempted to reveal the genes or pathways regulating floral degeneration in these species; however, no clear patterns have yet emerged. A possibility is that senescence and PCD are regulated posttranscriptionally, as argued by Thomas et al. (2003). Perhaps a complex network of both transcriptional and posttranscriptional control is involved, as is found in other fundamental cellular processes such as the cell cycle. If the underlying ethylene-independent lifespan control in ethylene-sensitive species is common to ethylene-insensitive species, then models such as *Arabidopsis* and *Brassica* or tomato and petunia may serve as better examples to investigate these control networks. Langston et al. (2005) used this approach to study DNA fragmentation in petunia, showing that the ethylene induction of a 43-kDa nuclease (PhNUC1) was delayed in 35S:etr1-1 plants but not eliminated. Likewise, in *Arabidopsis*, transcriptome and perhaps proteome analysis of petal senescence in etr1-1 lines may be a fruitful line of enquiry. However, if ethylene-independent regulation turns out to be species specific, then it is important to continue

work with the diverse species currently being studied to appreciate the range of networks employed.

Another important question is whether the same signal differentially regulates PCD in floral organs. In some cases the answer is yes: for example, in tobacco, ethylene regulates petal senescence (Rieu et al., 2003); however, at the same time ovary tissues continue to develop. So how is a primary signal such as ethylene transduced to ensure the coordinated life and death of different floral organs? Presumably, this is through differential signal translocation or differential signal perception. Petal margins often degenerate before the center and cross sections of developing petals reveal that while the epidermal cells are still functional, mesophyll cells have largely degenerated even before the flower is fully open (Wagstaf et al., 2003). So, is there a gradient of a diffusible signal, or of receptors or other intracellular mediators of the cell death signal? In some cases, this signaling differential is very distinct: in the *Arabidopsis* gfa2 mutant, synergids fail to undergo PCD, but antipodal PCD is not affected (Christensen et al., 2002). Ethylene is not the only PGR-stimulating PCD in floral organs: some links to other PGRs are reviewed in Wu and Cheung (2000). Mutation of gibberellic acid biosynthetic genes anther ear 1 and dwarf results in failure to abort stamens on maize female flowers. Mutation of a gene associated with brassinosteroids (TS) ("tasselseed") results in feminization of male flowers, and application of jasmonate (JA) enhances petal senescence in some species (Porat and Halevy, 1993), although this effect may be indirect through ethylene signaling. Elevating cytokinin levels in petunia delayed flower senescence; however, this may also be indirect through changes in sugar transport (Lara et al., 2004). So are all these PGRs involved in floral PCD in all species? Or are there important quantitative or even qualitative species-specific differences in their effects? Perhaps metabolomic approaches to measure endogenous levels of PGRs, coupled with a more extensive use of mutants, may begin to address these questions.

5.14 PCD mechanism in floral organs

van Doorn and Woltering (2005) have recently categorized plant PCD into three types: apoptotic, autophagic, and neither apoptotic nor autophagic. In animal cells, four types of apoptosis have been described (Orrenius et al., 2003), three of which involve cytochrome *C* release from the mitochondrion controlled by a family of proteins (Bcl-2) that interact with the mitochondrial membrane to facilitate or inhibit this process. Cytochrome *C* then activates a family of cysteine aspartate–specific proteases (caspases), which both regulate and affect PCD. Apoptotic PCD in animals is characterized by cytological features, including chromatin and nuclear condensation and marginalization followed by DNA fragmentation into nucleosomal units known as DNA laddering, nuclear blebbing, and formation of membrane inclusions known as apoptotic bodies (Cohen, 1993). The apoptotic bodies are then engulfed by neighboring living cells. In the tapetum and pollen tubes, there is compelling evidence to support an important role for the mitochondrion and involvement of caspases. This suggests a mechanism similar to animal apoptosis, although caution must be exercised in drawing too close a parallel, as engulfment of cellular remains by other cells does not occur in plants (van Doorn and Woltering, 2005). Following its nutritive role during pollen development, the tapetum degenerates. This is characterized by chromatin condensation in *Lobivia rauschii* and *Tillandsia albida* (Papini et al., 1999), and by DNA fragmentation in barley anthers (Wang et al., 1999).

PCD was also studied in the petals of *Antirrhinum majus*, *Argyranthemum frutescens*, and *Petunia hybrida*, using DNA degradation and changes in nuclear morphology as parameters by Yamada et al. (2006). The petals exhibit loss of turgor (wilting) as a visible symptom of PCD. DNA degradation, as shown on agarose gels, occurred in all species studied, prior to visible wilting. The number of DNA masses in all the petals of a flower, determined by flow cytometry, markedly increased in *Argyranthemum* and *Petunia*, but decreased in *Antirrhinum*. Many small DNA masses were observed in *Argyranthemum* and *Petunia*. The surface of each small DNA mass stained with the lipophilic fluorochrome 3,3′-dihexyloxacarbocyanine iodide (DiOC6), indicating that these masses were surrounded by a membrane. In *Antirrhinum*, in contrast, the chromatin fragmented into several small spherical clumps that remained inside a large membranous structure. Nuclear fragmentation, therefore, did not occur in *Antirrhinum*, whereas nuclear fragmentation possibly was a cause of the small DNA masses in *Argyranthemum* and *Petunia*. It is concluded that at least two contrasting nuclear morphologies exist during PCD. In the first, the chromatin fragments inside the nucleus, not accompanied or followed by nuclear fragmentation. In the second, a large number of DNA masses were observed each enveloped by a membrane. The second type was probably due, at least partially, to nuclear fragmentation (Yamada et al., 2006).

5.15 PCD functional categorization

A functional categorization of PCD can be made on the basis of the fate of the cell contents. Remobilization is central to leaf, sepal, and petal senescence (Thomas et al., 2003) and in a different way also to tapetal PCD. But endothecium, synergid, antipodal cell, or pollen-tube PCD is the selective death of unwanted cells. In green tissues, the chloroplast is seen by some (Thomas et al., 2003) as the key participant in the senescence process, and an early sign of senescence in green tissues is conversion of chloroplasts to gerontoplasts. Sepals, the floral organ that most closely resembles leaves, senesce in a similar way: in broccoli, sepal chlorophyll degradation is the first visual sign of senescence (Page et al., 2001). Petals are, however, not usually green, and an early step in their development is a conversion of chloroplasts to chromoplasts. This conversion has been compared with the chromoplast/gerontoplast transition (Thomas et al., 2003) with the inference that petals are most similar to senescent leaves. This agrees with the very early cell death seen in flowers (Wagstaff et al., 2003) presumably associated with nutrient remobilization. However, in-silico comparison of transcriptome changes in senescent *Arabidopsis* leaves and petals indicates that 25–30% of genes share similar patterns of expression. At a subcellular level, morphological changes to subcellular compartments during PCD are shared by many different cell types and tissues (Rogers, 2005). VPEs are found in leaves, roots, and flowers; ricinosomes are seen in seed and petal tissues; and caspase activity is detected in pollen tubes undergoing SI, and in many nonfloral tissues during natural senescence and also during pathogen responses (Sanmartín et al., 2005).

Progress in our understanding of PCD in plants has been rapid in the last 10 years, but the key regulators of some types of floral organ senescence, such as petal senescence in ethylene-insensitive species, remain obscure, except gladiolus (Arora and Singh, 2006). It is also unclear whether regulation of petal senescence and PCD in these species is similar or divergent. The latter is an important question to resolve before a good model for these species

Table 5.1 Comparison of signaling and possible mechanisms of PCD in floral organs

Floral organ	Intercellular signals	Mechanism for PCD	References
Sex organ abortion	GA and brassinosteroids	?	Wu and Cheung (2000)
Tapetum in cytoplasmic male sterility lines	Mitochondrial dysfunction	Cytochrome *C* release followed by loss of mitochondrial function	Balk and Leaver (2001)
Synergids	Pollination in some species	Requires mitochondrial function	Christenson et al. (2002)
Petal senescence	Ethylene in some species	Calcium/phosphate signaling, ROS increases	Porat and Halevy (1993), Kinoshita et al. (1999)
	Jasmonate (via ethylene)	Activation of vacuolar lytic enzymes through vacuolar-processing enzyme (caspase 1 activity)	Orzaez et al. (1999), Schmid et al. (1999)
	Cytokinin (via sugar transport)	Activation of vesicle bound proteases, vacuolar leakage	Wagstaff et al. (2003), Lara et al. (2004)
Pollen tube	During self-incompatibility	Increased calcium, cytochrome *C* release and caspase-3 activity	Thomas and Franklin (2004)

can be developed. Table 5.1 shows the comparison of signaling and possible mechanisms of PCD in various floral organs.

5.16 A proteomic analysis of plant PCD

Despite the fundamental importance of PCD in plants, comparatively little is known about the molecular mechanisms of plant PCD. Plant genomes do not contain obvious homologs to the key animal cell death proteins such as the Bcl-2 family proteins (Arabidopsis Genome Initiative, 2000), although a family of genes related to mammalian caspases has been identified in plants (Uren et al., 2000). Biochemical studies of plants have been able to establish a causal role for events such as the translocation of cytochrome *C* from the mitochondria to the cytosol (Balk et al., 1999; Sun et al., 1999; Zhao et al., 1999; Xu and Hanson, 2000). In an attempt to identify key plant PCD genes that may function universally during different types of plant PCD, Swidzinski et al. (2002) have previously undertaken a custom microarray analysis of gene expression during PCD in an *Arabidopsis* cell suspension culture. By identifying mRNA transcripts that changed in abundance following two unrelated PCD-inducing treatments (a brief, mild heat treatment for 10 min at 55°C and culture senescence), they have been able to discriminate between genes that may be common to a core plant cell death program and those that are specifically related to the inducing stimulus itself. While this study was successful in identifying several candidate genes whose common up- or downregulation during PCD may indicate a role for their products in plant PCD, it was restricted to elements of the PCD process that are transcriptionally regulated and ignored posttranscriptional and posttranslational regulation. Indeed, posttranslational events such as proteolytic cleavage and activation and modifications such as phosphorylation are key regulatory events in animal PCD (Reed, 2000). As a first step toward identifying such

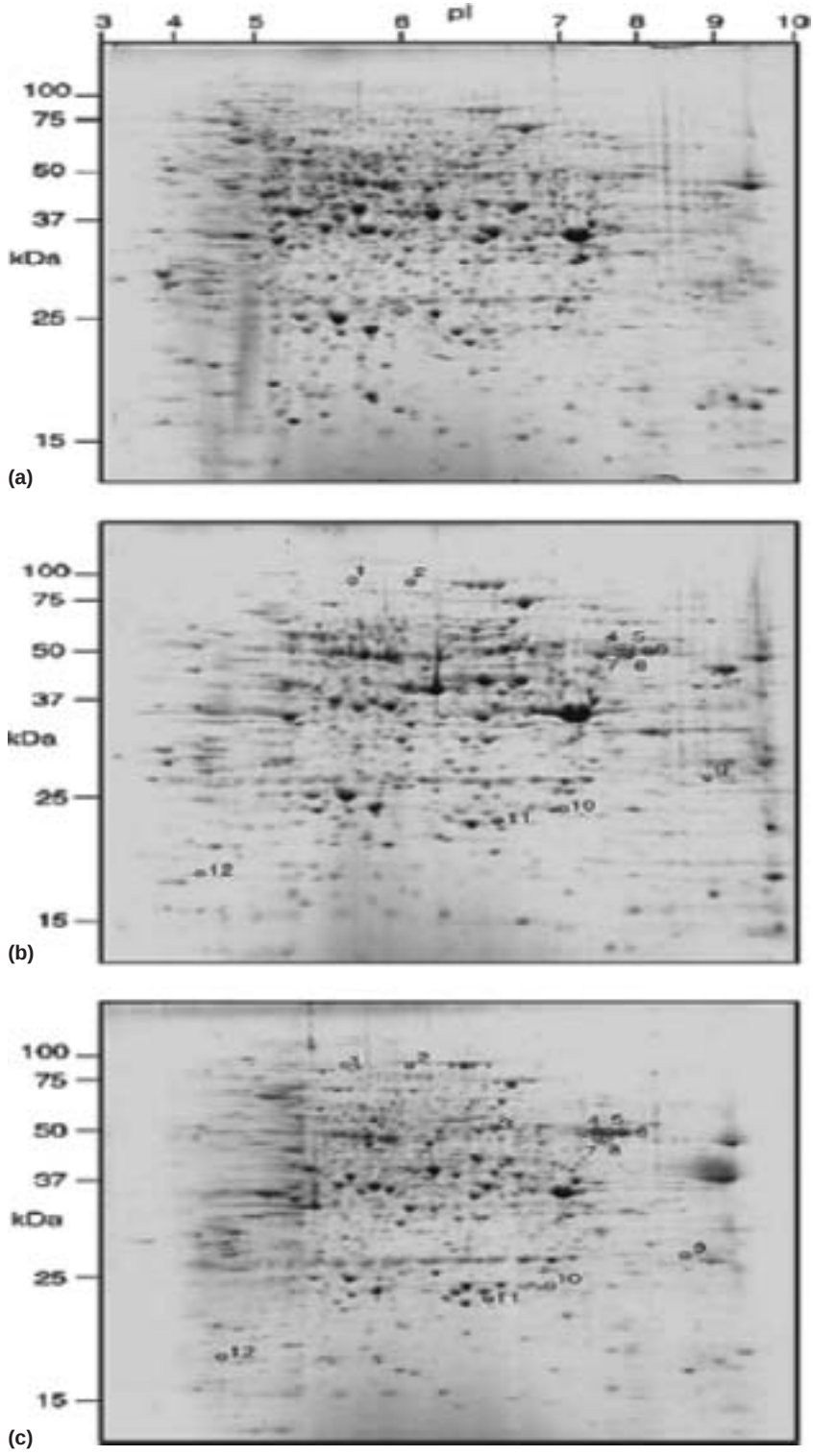

Fig. 5.4 Two-dimensional polyacrylamide gel electrophoresis analysis of total cellular protein from control cell cultures and cell cultures undergoing PCD. Spots (1–12) identified as being unique to and/or increased in relative abundance in both heat-treated and senescing samples undergoing PCD were excised, digested with trypsin, and analyzed by MS/MS. Each gel result was repeated in triplicate: (a) untreated, 6-day-old cultures; (b) 6-day-old cultures incubated for 10 min at 55°C and sampled immediately thereafter; (c) senescing, 13- to 14-day-old cultures (Swidzinski et al., 2004).

posttranscriptional mechanisms that function during plant PCD, a proteomic analysis of changes in total cellular protein content was performed during both heat- and senescence-induced PCD in an *Arabidopsis* cell culture. Both PCD systems were accompanied by a decreased protein content and an increased proteolytic activity. Analysis of two-dimensional gel electrophoresis displays of proteins revealed 11 proteins whose abundance (relative to total protein) increased following both treatments. The relative increase of these proteins in both heat- and senescence-induced PCD system suggests that they may play a general role in the plant cell death program (Swidzinski et al., 2002; Fig. 5.4).

5.16.1 Proteomic analysis of heat- and senescence-induced PCD

To identify proteins that are important in the PCD pathway, Swidzinski et al. (2002) fractionated equal amounts of protein from control cells, from heat-treated cells, and from senescent cells using two-dimensional gel electrophoresis. Sets of protein spots that increased in relative abundance (PCD/control) of at least twofold in comparison to the control in three replicate gels were identified. The increase in these proteins was statistically significant. From these sets, a subset of proteins that increased in abundance in both the heat-treated cells and the senescent cells was identified. Twelve protein spots were commonly increased in relative abundance in both treatments relative to the control, healthy cell cultures. These spots were excised from the gel, digested with trypsin, and the proteins identified using tandem MS/MS mass spectrometry. Four of these spots are isoforms of catalase, while several, including lipoamide dehydrogenase, the voltage-dependent anion channel protein Hsr2, and MnSOD are mitochondrial proteins. In addition to an EP1-like glycoprotein and a protein of unknown function, they also identified an aconitase protein that has previously been demonstrated to be present in *Arabidopsis* mitochondria but may also be present in the cytosol (Millar et al., 2001). The aconitase spot was increased in relative abundance by a factor of 2.9 in the senescence-induced PCD cells. In addition, the appearance of multiple spots that are the product of the same gene, suggesting that posttranslational modification of these proteins had occurred. Spots 4 and 5 both are encoded by the same catalase gene, *At1g20620*, and spots 10 and 11 are both products of the same gene, *At3g10920*, encoding MnSOD.

5.16.2 Relative increases in antioxidant enzymes are associated with plant PCD

The increased relative abundance of four catalase isoforms and two forms of mitochondrial MnSOD in both heat- and senescence-induced PCD is consistent with the observation that oxidative stress is implicated in the induction/execution of PCD (Swidzinski et al., 2002; Hildeman et al., 2003). Previous studies have shown that transgenic tobacco plants with reduced catalase levels show increased susceptibility to stress conditions (Willekens et al., 1997) and are hyperresponsive to pathogen attack (Mittler et al., 1999), indicating that this enzyme plays a central role in antioxidant defense. The identification of two isoforms of the same protein suggests that posttranslational modification of MnSOD may be important during plant PCD, and that perhaps such modifications occur only under severe conditions of oxidative stress, that is, those sufficient to cause PCD. This may be particularly important in preventing widespread mitochondrial damage during the initiation and execution of PCD,

since maintenance of mitochondrial function may be required during this time. Changes in levels of ROS and oxidative stress are often observed in association with plant programmed cell death. However, it remains unclear whether increased ROS are generated actively as a signal to trigger the PCD pathway, or whether ROS are a byproduct of the stress condition that induces PCD. If the former possibility is correct, then one would expect to see a downregulation of antioxidant enzymes such that the ROS signal is not quenched. Such a downregulation has been observed during developmental PCD in barley aleurone cells (Fath et al., 2001).

5.16.3 Specific mitochondrial proteins are associated with PCD

It is remarkable that of the eight gene products whose relative abundance increased following the induction of PCD, four are targeted to the mitochondrion. This may either imply that mitochondrial proteins are particularly important during PCD or that proteins within the mitochondrion are protected from the proteolytic degradation that is occurring elsewhere in the cell. If the latter is true, then one would expect to see an increase in abundance of all mitochondrial proteins relative to total cellular protein. The abundance of several other mitochondrial proteins by Western blotting was assayed by Swidzinski et al. (2002). The following proteins, representing different submitochondrial compartments, were analyzed: porin/VDAC (outer membrane), adenine nucleotide translocase (inner membrane), fumarase (matrix), and the E1a subunit of pyruvate dehydrogenase complex (matrix). The increase in relative abundance of VDAC in both heat-treated and senescent cells observed on two-dimensional gels was confirmed, but other mitochondrial proteins did not follow the same pattern. For example, E1a is almost undetectable in heat-treated cells but appears to be increased during senescence. Conversely, fumarase levels are increased in the former and decreased in the latter. It appears that not all mitochondrial proteins are maintained during PCD, but specific mitochondrial proteins (including superoxide dismutase (MnSOD), a voltage-dependent anion channel (VDAC) Hsr2, aconitase, and lipoamide dehydrogenase may play important roles in the PCD pathway. The increased relative abundance of lipoamide dehydrogenase, a subunit that is a part of several mitochondrial multienzyme complexes including pyruvate dehydrogenase complex (PDC) and 2-oxoglutarate dehydrogenase complex (2-OGDC) (Lutziger and Oliver, 2001), is interesting given that other subunits of these complexes, such as the E1a subunit of PDC, decrease in abundance during heat-induced PCD. This suggests the increased lipoamide dehydrogenase content is not related to the function of mitochondrial dehydrogenase complexes but may reflect an alternative function for lipoamide dehydrogenase. One possibility is that changes in the redox state of lipoamide may form part of a redox signaling mechanism.

5.16.4 Identification of a potential cell-to-cell PCD signaling mechanism

One of the protein spots that increased in relative abundance during heat- and senescence-induced PCD was identified as an EP1-like protein. This protein is 51% identical and 67% similar to an extracellular glycoprotein, EP1, initially characterized in carrot suspension

cells (van Engelen et al., 1991). The protein identified in this study bears homology (40–60% similarity) to S-like glycoproteins and the S-like domain of receptor proteins kinases from several species, including *Arabidopsis*. Since *Arabidopsis*, like carrot, does not possess a genetic self-incompatibility system (Bi et al., 2000), the EP1-like protein may be involved in other receptor kinase activation pathways and signal transduction. The encoded EP1-like protein is predicted to be a part of the secretory pathway (Emanuelsson et al., 2000) and therefore may be secreted in *Arabidopsis* cell cultures undergoing PCD as part of a cell-to-cell signaling mechanism.

Swidzinski et al. (2004) identified a number of proteins that are increased in relative abundance during PCD-induced in an *Arabidopsis* cell suspension culture by two independent means. These proteins appear to be maintained in the face of general and extensive protein degradation and therefore may be required to allow PCD to proceed. Several of these proteins show evidence of posttranslational modifications, which may alter their properties for a PCD-specific function. While the identified antioxidant proteins are most probably a response to the stress of the inducing stimuli rather than being related directly to the PCD process, plausible PCD-related roles for several of the other proteins can be hypothesized. It is particularly intriguing that they identified several mitochondrial proteins since the mitochondrion has been established to be at the heart of the PCD pathway in animals (Kroemer and Reed, 2000). These mitochondrial proteins may be involved in redox signaling (lipoamide dehydrogenase and aconitase) that triggers PCD or in the release of proapoptotic mitochondrial proteins into the cytosol (VDAC). They also identified an extracellular glycoprotein that bears sequence similarity to receptor kinases and may therefore be part of a signaling mechanism that transmits a "death signal" between cells. Such a mechanism may be important in maximizing the efficiency of a localized death lesion to minimize pathogen spread or transmission of oxidative insults. The identification of such a receptor-based pathway of PCD would not only have direct biological significance, but would also constitute a much needed research tool that would allow the precise induction of PCD in the absence of a stress stimulus. Such an approach would allow a more definitive identification of genes and proteins that play a role in plant PCD. This study demonstrates the utility of the proteomic approach in addressing a biological system in which there is little prior knowledge to form the basis of more hypothesis-driven studies. Proteomic analysis identified a number of proteins that are putatively involved in plant PCD and have provided a foundation for further functional studies to examine the precise roles and functions of these proteins.

5.17 Conclusions and future directions

The examples of developmental PCD in the plants discussed, illustrate a diversity of datasets but also some fundamental differences between the different cell fates and PCD mechanisms. It can be asked, how useful is the animal model of apoptosis or autophagy in explaining plant PCD mechanisms? In common with most animal PCD systems, a signal extrinsic to the cell, often hormonal is usually involved. However, there is no one PGR which induces PCD, although ethylene, GA, and brassinosteroids appear in more than one system. There are few clear-cut examples of completely autonomous PCD, although perhaps the root cap comes close. Another interesting issue is how cells adjacent to cells undergoing PCD protect

themselves from the "death signals." Data from ethylene signaling in the endosperm (Gallie and Young, 2004) indicate that at least in some systems this may be achieved by a difference in sensitivity. However, in the TE system another mechanism seems to be operating. Here, there is evidence for an inhibitor of proteasome-mediated protein degradation which is released into the apoplastic space (Endo et al., 2001) and which protects living cells from the hydrolases released during TE PCD. Vacuolation accompanies PCD in more than half of the examples reviewed, and in generally rupture of the vacuole coincides with release of hydrolases into the cytoplasm. This suggests a possible model (Fig. 5.5) that would account for at least some of the systems reviewed. In this model an external signal activates increases in cytoplasmic calcium, which in turn stimulates the fusion of small vacuoles derived either from the ER or the golgi to form a large vacuole. This vacuole accumulates hydrolytic enzymes. Its collapse, resulting either from ROS accumulation, or activation of a proteolytic cascade, releases hydrolases into the cytoplasm. The hydrolase release results in organellar breakdown and macromolecule degradation ending in cell death. This model has clear parallels to animal autophagy although the vacuole rather than the lysosome is the primary organelle involved.

There are many open questions in the field of PCD in plants. However, we have most of the experimental tools in place and we should be able to answer these questions in the near future. Identifying more PCD genes in plants and placing them along the different PCD

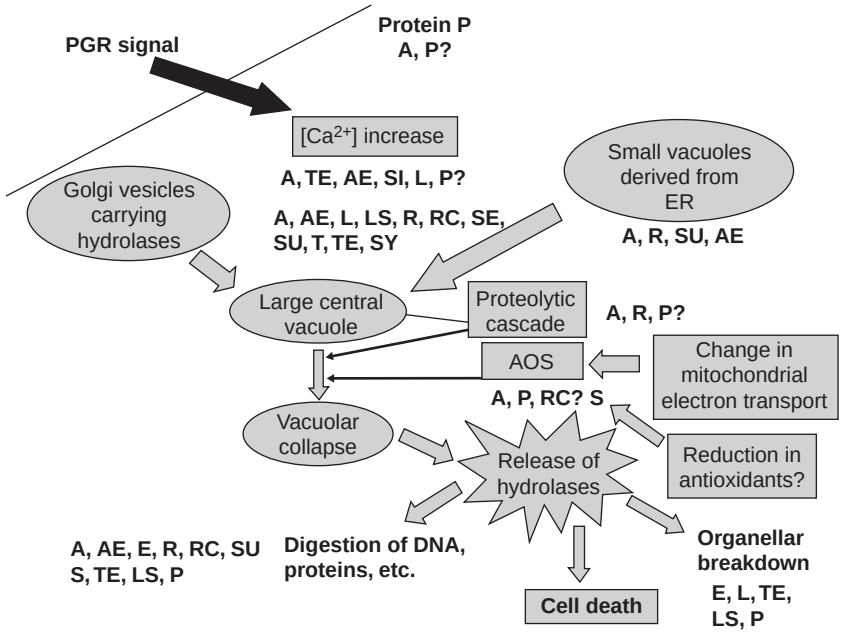

Fig. 5.5 Model showing major signals (black arrows) and cytological/biochemical events (open arrows) during autophagic-type PCD in plants. Cell types/tissue in which these features have been reported are indicated as follows: A, aleurone cells; AE, aerenchyma; L, leaf sculpting; LS, leaf senescence; P, petal senescence; R, *Ricinus* endosperm; RC, root cap; S, starchy endosperm; SE, supernumerary embryos; SI, pollen tube during SI interaction; SU, suspensor; SY, synergids; T, tapetum; TE, tracheary elements.

pathways should be our first priority. These studies would lead to elucidating the relationship(s) between different PCD pathways in plants (developmental, pathogen-derived, and abiotic stress-associated), and to a broader understanding of PCD in plants. As described earlier, the benefits to agriculture should more than compensate for the expenses spent on research related to PCD in plants.

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Chapter 6

Ethylene Perception and Gene Expression

Willis O. Owino and Hiroshi Ezura

6.1 Introduction

Fruits, vegetables, and flowers are economically important horticultural products liable to postharvest deterioration and thus require appropriate postharvest handling techniques to lengthen their shelf life while maintaining quality. An understanding of the key control points of the physiological processes preceding senescence is necessary in developing useful strategies for delaying postharvest deterioration in these plant products.

The phytohormone ethylene is known to regulate multiple physiological and developmental processes in plants, such as leaf and flower senescence, fruit ripening, organ abscission, and growth transition from vegetative phase to reproductive phase, and is also involved in the reactions of plants to abiotic and biotic stresses (Abeles et al., 1992; Ogawara et al., 2003; Guo and Ecker, 2004; Chen et al., 2005). Ethylene perception is a key event during such physiological responses. Extending shelf life by delaying the biosynthesis and/or minimizing the action of the plant hormone ethylene has been an attractive target area of study for postharvest physiologists. This technique has the potential to reduce damage and postharvest loss of horticultural products while increasing the shipping range and market area. This chapter outlines the major advances in our understanding of ethylene perception in fruits, vegetables, and flowers in the recent past.

6.2 Background on ethylene perception

After ethylene has been synthesized, it is perceived and the signal transduced via a transduction machinery to trigger specific biological responses. Many key components of the ethylene signal transduction pathway were identified from a simple genetic screening of the model plant species *Arabidopsis thaliana* that made use of ethylene's effect on dark-grown seedlings known as the "triple response." The triple response is characterized by the inhibition of hypocotyl and root elongation, a thickening of hypocotyl, and an exaggerated apical hook. (An illustration of the "triple response" using dark-grown melon seedlings is shown in Fig. 6.1.) Taking advantage of the triple responses, populations of mutagenized *Arabidopsis* were screened for seedlings that displayed an altered triple-response phenotype, and this approach resulted in the identification of several ethylene-insensitive mutants. These mutants include those that are insensitive to the ethylene receptors *etr1* (ethylene

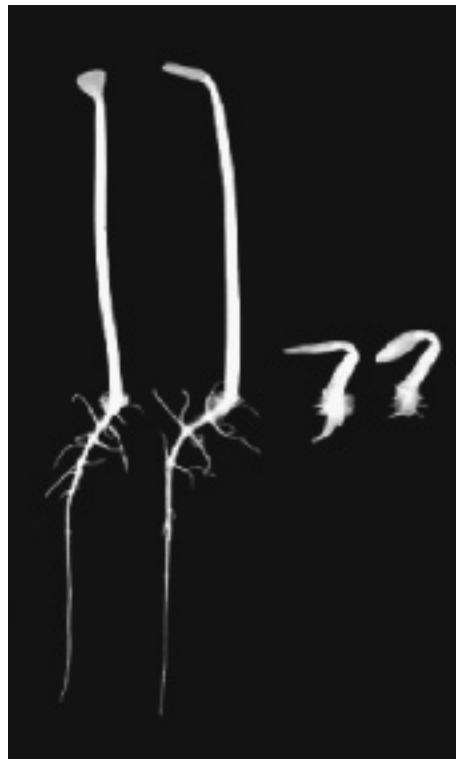

Fig. 6.1 The triple response of dark-grown melon seedlings to ethylene. Wild-type seedlings grown in the absence (left) or presence (right) of ethylene.

response) (Bleecker et al., 1988; Chang et al., 1993) and *etr2* (Sakai et al., 1998). Cloning and characterization of genes disrupted in these mutants have defined a mostly linear pathway for ethylene signal transduction leading from initial hormone perception to transcriptional regulation.

In *Arabidopsis*, ethylene perception initiates with the binding of ethylene to a family of five receptors, namely, ETR1, ERS1, ETR2, EIN4, and ERS2. ETR1 was the first member of the receptor family identified, and has been characterized in the most detail. Ethylene binding is mediated by a copper cofactor (Rodriguez et al., 1999) that is provided to the receptors by the copper transporter RAN1. All of the encoded receptor proteins show similarity to bacterial two-component His kinases, which allows bacteria to adapt to changing environmental conditions (Chang et al., 1993; Hua et al., 1995, 1998; Sakai et al., 1998).

As with the two-component regulators in bacteria, the ethylene receptors can be divided into multiple functional domains including a sensor domain that consists of a transmembrane region responsible for ethylene binding (Schaller and Bleecker, 1995; Hall et al., 2000); a GAF domain of unknown function (GAF domains are ubiquitous motifs present in cyclic **g**uanosine monophosphate (cGMP)-regulated cyclic nucleotide phosphodiesterases, certain **a**denylyl cyclases, the bacterial transcription **F**actor FhlA, and hundreds of other signaling and sensory proteins from all three kingdoms of life) (Aravind and Ponting, 1997); a His kinase domain, and, in the case of *ETR* and *EIN4* type receptors, a receiver domain predicted

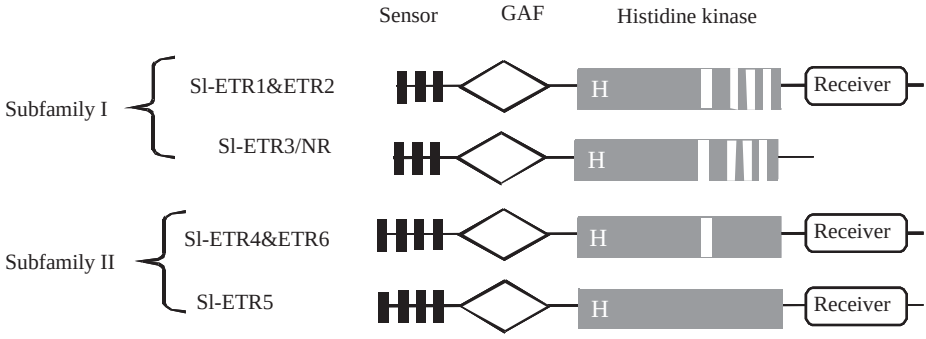

Fig. 6.2 Schematic representation of the tomato ethylene receptor proteins and their functional domain structures similar to *Arabidopsis*. The sensor domain contains three hydrophobic, transmembrane regions. Ethylene binding occurs within this amino terminal hydrophobic region. Subfamily II has a fourth membrane spanning domain. The GAF domain is conserved among a range of diverse group of proteins. Its function in ethylene signaling is unknown. There are five subdomains that define the catalytic core of His kinase domain. While subfamily I contains all of these subdomains, subfamily II lacks one or more of them.

to modulate the activity of a downstream factor (Chang et al., 1993; Hua et al., 1998; Sakai et al., 1998). Recent studies with bacterial two-component systems support an important role for receptor interactions in signal output. In plants, ETR1 and ERS1 receptors have been shown to form homodimers, while ERS-type receptors have been postulated to use the receiver domains of other receptors to form heterodimers with them (Schaller et al., 1995; Takahashi et al., 2002; Wang et al., 2003).

Based on distinguishing structural features and overall sequence similarity, the members of the ethylene receptor family can be divided into two subfamilies: subfamily I and subfamily II (Stepanova and Alonso, 2005). Subfamily I ethylene receptors have three transmembrane domains and a well-conserved histidine kinase domain. On the other hand, subfamily II receptors contain a putative signal peptide in addition to the three conserved transmembrane domains and a histidine kinase domain that lacks one or more elements that are necessary for catalytic activity. (A schematic representation of the tomato receptors structures similar to that in *Arabidopsis* is shown in Fig. 6.2.)

6.3 Ethylene perception in fruits and vegetables

Subsequent studies regarding ethylene perception have focused on the isolation and characterization of the receptor gene family from various plant species. However, in fruit and vegetable species, tomato has emerged as the most useful model to date, due to its commercial importance, ease of genetic manipulation, rapid life cycle, year-round nonseasonal greenhouse fruit production, well-characterized single gene-ripening mutants such as never ripe (*nr*), nonripening (*nor*), ripening inhibitor (*rin*), and green ripe (*gr*) and the availability of detailed genetic maps, EST collections, microarray chips, and full-length cDNA collections (Alexander and Grierson, 2002; Barry et al., 2005; Barry and Giovannoni, 2006; Klee, 2006). Consequently, much of our understanding of ethylene perception in fruit species comes from studies on tomato. The tomato has been renamed *Solanum lycopersicum* (formerly *Lycopersicon esculentum*), and this had led to the renaming of its genes.

The involvement of ethylene and its receptors in the ripening of climacteric fruits has been reaffirmed in several fruits, even though it has also emerged that the role played by ethylene is not exclusive, since ethylene-independent pathways are also involved in the ripening process (Giovannoni, 2004). There appear to be significant differences in the way ethylene signaling is regulated in *Arabidopsis* and tomato. But the building blocks of ethylene signal transduction are very similar between the two species. A family of six genes encoding tomato ethylene receptors (LeETR1-6/SlETR1-6) has been isolated and characterized and the predicted structures are similar to *Arabidopsis* (Fig. 6.2) (Alexander and Grierson, 2002; Klee and Tieman, 2002; Klee, 2002, 2004, 2006). The proteins encoded by these genes are structurally diverse and, at the most, are less than 50% identical. The mRNA expression patterns vary among the tomato *ETR1* homologs. *LeETR1* is expressed constitutively in all tissues examined. *LeETR2* is expressed at low levels in all tissues with induction in seeds before germination and downregulation in elongating seedlings and senescing leaf petioles. *NR* (*LeETR3*) mRNA is upregulated in ovaries and ripening fruit. The *LeETR4* mRNA is present at high levels in fruit but is low in vegetative tissues. The *LeETR5* expression pattern is similar to *LeETR4*, but absolute mRNA levels are lower. The *LeETR6* mRNA is abundantly expressed in flowers and fruits and less in vegetative tissues.

Other than tomato fruit, the ethylene receptors have been isolated in several climacteric and nonclimacteric fruits, and exhibits different expression patterns during ripening. The melon fruit is second to tomato fruit when it comes to research work carried on ethylene perception. Melon fruit is an ideal fruit for these studies due to the fact that its development has three distinct stages: phase I, II, and III; the flesh, embryo, placenta, and seeds are well ordered; the fruit development can be clearly divided into ethylene-insensitive and ethylene-sensitive stage, and the developing fruit has a lower sensitivity to ethylene than does the ripening fruit (Gillaspy et al., 1993; Takahashi et al., 2002). In muskmelon, *Cm-ERS1* mRNA increased slightly in the pericarp of fruit during ripening, followed by a marked increase of *Cm-ETR1* mRNA, which paralleled climacteric ethylene production. The increase of *Cm-ERS1* mRNA at a low concentration of ethylene before the increase of *Cm-ETR1* mRNA and ethylene production indicates that *Cm-ERS1* may be sensitive to a much lower concentration of ethylene, while *Cm-ETR1* may be involved in the response at a high concentration of ethylene (Sato-Nara et al., 1999). Studies carried out to examine the temporal and spatial expression pattern of Cm-ERS1 protein, during fruit development, revealed that a posttranscriptional regulation of Cm-ERS1 expression affects stage- and tissue-specific accumulation of the protein (Takahashi et al., 2002). The melon receptor CmERS1 was localized at the endoplasmic reticulum and its topology indicates that there are three membrane-spanning domains, with its N-terminus facing the luminal space and the large C-terminal portion being located on the cytosolic side of the ER membrane (Fig. 6.3) (Ma et al., 2006a). The melon subfamily II ethylene receptor, *Cm-ETR2* mRNA, exhibits earlier accumulation compared to *Cm-ETR1* during ripening, and its transcript accumulation increased during melon ripening, and declined in parallel with a reduction in ethylene production. Furthermore, the *Cm-ETR2* mRNA was induced by ethylene treatment and inhibited by 1-MCP (Owino et al., unpublished results).

The expression of two ethylene receptor genes in passion fruit (*Passiflo a edulis*), *PeETR1* and *PeERS1*, did not change significantly during ripening. However, the levels of *PeETR1* and *PeERS1* mRNA were much higher in arils than in seeds (Mita et al., 1998).

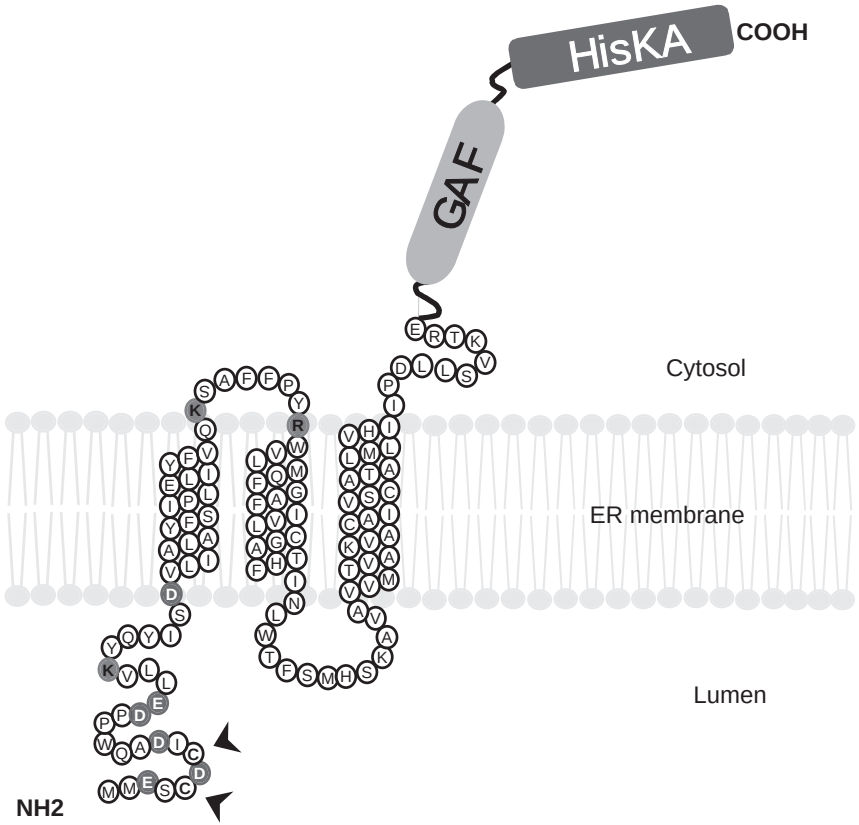

Fig. 6.3 A proposed model for CmERS1 topology on the ER membrane. Based on membrane fractionation, GFP imaging, protease protection assay, and glycosylation analysis, we propose that CmERS1 is predominantly localized to the ER and spans the membrane three times with its N-terminus facing the luminal space and its C-terminus lying on the cytosolic side (Ma et al., 2006a).

High levels of *PeERS2* mRNA were detected only in the arils of ripe purple fruit. Although the expression of *PeERS2* mRNA was enhanced during ripening, a markedly high level of *PeERS2* mRNA was detected only in the arils of ripened purple passion fruit, and no ripening-regulated expression was apparent in seeds. Exposure of mature green fruit to ethylene increased the levels of *PeERS2* mRNA, suggesting that PeERS2 might play a role in repressing ethylene responses at later developmental stages after fruit ripening has been completed (Mita et al., 2002).

Ethylene perception has also been described to be involved in apple fruitlet abscission and early development (Cin et al., 2005). The apple (*Malus domestica*) *MdETR1* and *MdERS1* gene expression patterns were tissue specific, with *MdETR1* transcripts being abundant in the peduncle, abscission zone, and seed than in the cortex of early developing fruits (nonabscising fruitlets), even though the expression always remained at a steady-state level. The *MdERS1* transcripts increased throughout shedding in all tissues of abscising fruitlets, indicating a possible role for this ethylene receptor in abscission. An increase in ethylene evolution and/or sensitivity at the abscission zone level would regulate the

expression of genes encoding specific cell wall hydrolases, leading to abscission zone cell separation and to fruitlet shedding.

The avocado (*Persea americana*) *PA-ERS1* mRNA increased gradually from the day of harvest, and did not change significantly until the climacteric peak when it was hyperinduced. 1-MCP however suppressed the accumulation of *PA-ERS1* to basal levels suggesting that the stimulated induction of *PA-ERS1* at the climacteric peak maybe a mechanism by the avocado fruit to dissipate the high levels autocatalytic ethylene (Owino et al., 2002).

In peach (*Prunus persica*), the expression of *Pp-ETR1* appeared to be constitutive and ethylene independent during fruit development and ripening, while *Pp-ERS1* transcripts increased during fruit ripening and its expression appeared to be upregulated by propylene treatment (Rasori et al., 2002). Application of the ethylene antagonist, 1-MCP, delayed fruit ripening, ethylene evolution, and concurrently downregulated *Pp-ERS1*, while *Pp-ETR1* transcription was unaffected. 1-MCP action was rapidly abolished after moving fruits to air, when a rapid stimulation of ethylene evolution and a concurrent increase of *Pp-ERS1* mRNAs were observed.

Cold treatment of late-season pear (*Pyrus communis* cv. Passe-Crassane) fruit leads to a gradual increase in ethylene production and a commensurate increase in ethylene receptor mRNA expression (El-Sharkawy et al., 2003). The *Pc-ETR1a* mRNA accumulation was upregulated by cold and during ripening, whereas *Pc-ERS1a* and *Pc-ETR5* were less affected by cold treatment, but all increased during postcold treatment, ethylene-dependent ripening. A sharp peak of *Pc-ETR1a* and *Pc-ERS1a* mRNA accumulation was observed during ripening in the early-season pear cultivars, in contrast to the gradual increase seen in late-season pear cultivar, Passe-Crassane (PC). A more pronounced difference between early-season cultivars and late-season cultivar PC was seen in the behavior of *Pc-ETR5* transcript accumulation. Transcript levels for *Pc-ETR5* diminish sharply before and during the ethylene climacteric and ripening of early-season pear fruit, whereas in late-season cultivar they increase sharply. This suggests that a decrease in the expression of a negative regulator could result in an increase in ethylene sensitivity early in the ripening phase of early fruit development. However, given the potential for redundancy in the ethylene receptor family, it remains to be determined whether reduced levels of *Pc-ETR5* affect the overall ethylene sensitivity of early-season pear fruit.

Three ethylene receptors—*DkERS1*, *DkETR1*, and *DkETR2*—have been isolated and their expression determined during ripening of persimmon (*Diospyros kaki*) fruit (Pang et al., 2006). The *DkETR1* mRNA is constitutively expressed during all stages of fruit ripening and is ethylene-independent. Conversely, *DkERS1* and *DkETR1* mRNA levels correlated with ethylene production during fruit development and ripening and were induced by ethylene. The DkERS1 protein decreased gradually prior to fruit maturation and reached its lowest level at the ripening stage when ripening-related ethylene was produced, suggesting the involvement of DkERS1 in ethylene perception during fruit ripening.

In contrast to the great deal of information available regarding the ethylene receptors in climacteric fruits, much less is known about nonclimacteric fruits. At present, no single growth regulator appears to play a positive role analogous to the role played by ethylene in the ripening of climacteric fruits. Nonclimacteric fruits are also able to synthesize ethylene, and in some cases, it has been shown that ethylene can hasten the postharvest deterioration. However, in spite of many efforts, no results have been obtained that can demonstrate a clear relation between ethylene and the ripening of these fruits. Three ethylene receptors

were isolated and expression patterns determined in strawberry (*Fragaria ananassa*) fruits (Trainotti et al., 2005). The *FaEtr1* mRNA was low in flowers, but showed an increase in the small green fruits and a subsequent decrease in the large green fruits that was followed by a steep increment, which continued throughout the ripening phase. The *FaErs1* mRNA was very high in flowers but steadily decreased to reach a minimum in the large green fruits. Afterward, it increased again till the ripening was completed. On the other hand, *FaEtr2* mRNA increased about threefold to reach a maximum in the white fruits. Afterward, although a slight decrease was observed, the transcript amount remained high in the red fruits at well over twice that of the small green fruits. The *FaEtr1* and *FaEtr2* genes were more responsive to ethylene in the white fruits, while *FaErs1* was highly responsive to ethylene at the red stage. This study suggested that ethylene receptors might have a physiological role in the ripening of nonclimacteric strawberries.

Even though citrus (*Citrus sinensis*) fruits are nonclimacteric, exogenous ethylene is able to stimulate ripening by accelerating respiration and inducing pigment changes of peel, chlorophyll degradation, as well as carotenoid biosynthesis. In young "Valencia" fruitlets, *CsERS1* expression was detected in fruits on tree, immediately after harvest, and was further induced in the subsequent days (Katz et al., 2004). The *CsERS1* expression was slightly induced by ethylene treatment and reduced by 1-MCP treatment in young fruitlets. The *CsETR1* expression was constitutive in young fruitlets, but was not affected by detachment from the tree and was ethylene-independent. In mature fruit, the expression of both *CsERS1* and*CsETR1* genes was constant and was not affected either by 1-MCP or propylene treatments. The differences in the expression of *CsERS1* between young fruitlets and mature fruit suggest that *CsERS1* may modulate the differential sensitivity to ethylene in fruitlets versus mature citrus fruit.

6.4 Ethylene perception in flowers

The postharvest quality of many flowers is limited by the increased synthesis and action of the plant hormone ethylene. Increased production of ethylene plays a role in the senescence or death of flower petals, abscission of plant parts including floral structures, and discoloration of harvested foliage. Abscised and senesced flowers show reduced visual impact, increase the incidence of infection from *Botrytis* and other saprophytic pathogens, thus diminishing the commercial value of flowers.

Just like fruits, flowers are categorized as being climacteric or nonclimacteric. In climacteric flowers such as carnations, *Gypsophila*, and orchids, senescence is accompanied by a sudden, transient increase in ethylene production and respiration, while treatment of nonsenescent flowers with ethylene rapidly induces petal senescence. In nonclimacteric flowers such as gladiolus, tulip, and iris, generally, no increases in ethylene production and respiration are apparent during flower senescence, and exogenous ethylene application has little or no effect on petal senescence (Serek et al., 2006). Several studies to date suggest that abscission and senescence of flowers may be triggered by the perception of endogenous ethylene by ethylene receptors. Abscission is a typical ethylene response induced through ethylene receptors and is influenced by mutations in ethylene receptors (Patterson and Bleecker, 2004). Therefore, investigations of ethylene receptors and associated signal transduction pathways are essential for understanding of ethylene perception in flowers.

Delphinium flowers are sensitive to ethylene, which is produced in the pistil and receptacle, and causes abscission of the sepals, posing a serious problem in marketing. *Delphinium hybrid* cv. Bellamosum flowers show a climacteric-like rise in ethylene production before the abscission of sepals, and sepals are abscised by exposure to exogenous ethylene. Gynoecia and receptacles are the main ethylene producers in flowers, and the sepals, which are most influenced by ethylene, are abscised after the peak in ethylene production (Ichimura et al., 2000). The rate of senescence of *Delphinium* florets seems to be well correlated to both ethylene production and levels of *Dl-ERS1*, suggesting that ethylene evolved by the florets is perceived by elevated levels of *Dl-ERS1* to cause senescence of florets in *Delphinium* (Kuroda et al., 2003). On the other hand, *Dl-ERS1-3* and *Dl-ERS2* mRNAs were upregulated by ethylene in *Delphinium* sepals, but did not change markedly during flower senescence (Tanase and Ichimura, 2006).

In geranium (*Pelargonium* × *hortorum*) flowers, the levels of *Ph-ETR1* and *Ph-ETR2* transcripts were not upregulated by exogenous ethylene and did not change during flower senescence. *Ph-ETR1* and *Ph-ETR2* were not affected by ethylene exposure in receptacles containing abscission zones (Dervinis et al., 2000).

With respect to the role of ethylene in flower opening of cut roses, it has been reported that flower opening is ethylene-influenced, and the effect of ethylene is cultivar-dependent, and that the cultivars can roughly be divided into three groups: opening inhibited, opening stimulated, and opening not affected by ethylene (Yamamoto et al., 1994). The level of mRNA for an ethylene receptor (RhETR2) in petals of rose (*Rosa hybrida*) flowers at the full-opening stage was two times higher in "Bronze" cultivar, which has a short floral life and sensitive to ethylene, than that in "Vanilla" cultivar, a cultivar less ethylene sensitive with a long floral life (Müller et al., 2000). While *RhETR2* expression varied during flower development and in response to ethylene, *RhETR1* and *RhETR3* exhibited differential expression during flower development and appeared to be rate limiting for ethylene perception and determinants of flower longevity. Expression of *RhETR1* was distinctly higher in "Bronze," than in "Vanilla." While expression of RhETR1 preceded the ethylene production by the flowers, abundance of the *RhETR3* transcript increased during flower senescence in "Bronze," indicating that the ethylene response system in rose flowers is composed of multiple receptor types with overlapping patterns of expression. In "Vanilla," a cultivar that has excellent flower longevity despite moderate ethylene production, expression of *RhETR1* and *RhETR3* was reduced. In another study involving two ethylene-sensitive cut rose flowers "Kardinal" and "Samantha," expression level of the three *ETRs—Rh-ETR1*, *RhETR3*, and *RhETR5*—was higher in "Samantha" than in "Kardinal," indicating that higher ethylene sensitivity of "Kardinal" is probably due to its lower expression level of ethylene receptors during the flower opening process (Tan et al., 2006). These results suggested that differences in flower life among rose cultivars—in an ethylene-free environment and in response to exogenous ethylene—may be due to differences in receptor expression levels. In "Samantha," *Rh-ETR5* was found to be constitutive and ethylene-independent, whereas *Rh-ETR1* and *Rh-ETR3* were induced by ethylene and suppressed by 1-MCP (Ma et al., 2006b).

In carnation flowers (*Dianthus caryophyllus*), the levels of *DC-ERS2* and *DC-ETR1* mRNAs in petals increased with an increase in ethylene production. However, ethylene treatment did not affect the levels of these mRNAs in petals. Furthermore, the levels of these mRNAs decreased independent of ethylene production in petals of flowers treated with DPSS (1,1-dimethyl-4-(phenylsulfonyl)semicarbazide, a putative inhibitor of abscisic

acid biosynthesis), which blocked ethylene production in the flowers. These results indicate that the *DC-ERS2* and *DC-ETR1* genes are not subject to positive regulation by ethylene, and the decrease in the levels of their mRNAs in senescing petals is caused by some unidentified senescence factor(s) other than ethylene (Shibuya et al., 2002).

In the cut flowers of "Seiko-no-makoto" (ethylene-sensitive cultivar) chrysanthemum (*Dendranthema grandifloru* (Ramat.) Kitamura), *DG-ERS1* mRNA was present in a large amount in the petals on day 0 (at the full-opening stage of flower) and its levels decreased markedly with the lapse of time in air or in response to a 12-h exposure to ethylene, although these were not evident in "Iwa-no-hakusen" chrysanthemum (ethylene-insensitive cultivar). *DG-ERS1* mRNA was present in a large amount in nonsenescent flower tissues of an ethylene-sensitive "Seiko-no-makoto" cultivar, but its mRNA level was very low in an ethylene-insensitive "Iwa-no-hakusen" cultivar. The observed difference between the two cultivars is probably related to the variation in sensitivity to ethylene between them, and suggest that the *DG-ERS1* gene and its resultant DG-ERS1 protein may be involved in the perception of ethylene signal in flower and leaf tissues of the cut chrysanthemum, especially those of "Seiko-no-makoto" (Narumi et al., 2005a).

Two ethylene receptor paralogous genes, *GgERS1a* and *GgERS1b*, have been isolated from gladiolus (*Gladiolus randiflo a* hort cv. Traveler), an ethylene-insensitive flower (Arora et al., 2006). The cDNA sequence indicated that both genes have almost exact similar sequence except that *GgERS1b* lacks 636 nucleotides, including first (H) and second (N) in the histidine kinase motifs, present in *GgERS1a*. The analysis of the genomic DNA sequences (4,776-bp nucleotide designated as *GgERS1* long DNA and 3,956-bp nucleotide designated as *GgERS1* short DNA) revealed that both sequences were identical except that *GgERS1* short DNA was devoid of an 820-bp long nucleotide segment in the first intron of *GgERS1* long DNA. These data suggested that each of the *GgERS1* genes was generated by duplication and splicing from different genomic DNA. The *GgERS1b* mRNA level decreased in petals during flower development, whereas the expression of *GgERS1a* mRNA was constitutive, however, with a high accumulation level, suggesting that high expression level of *GgERS1a* conferred the ethylene insensitive nature in petals of gladiolus. On the other hand, the sensitivity to ethylene might be regulated by *GgERS1b* expression.

6.5 Control of ethylene perception

Ethylene receptors have been postulated to function via the "receptor inhibition" model in *Arabidopsis* in which absence of ethylene results in active receptors and repression of ethylene responses (Hua and Meyerowitz, 1998). In the presence of ethylene, receptors switch to an inactive state, and responses such as the triple responses are observed. Attempts to control ethylene perception by decreasing ethylene sensitivity and improve postharvest quality have been carried out on various horticultural plant products using such approaches as antisense RNA or transformation with a gene coding for a mutant receptor that does not bind ethylene and which constitutively suppresses the normal ethylene responses.

An important aspect of the control of ethylene perception was identified by analysis of transgenic tomato plants with reduced expression of individual receptors. Antisense reduction in *LeETR4* expression resulted in earlier and accelerated fruit ripening and higher levels of lycopene accumulation (Tieman et al., 2000). Some of the antisense lines also synthesized significantly more ethylene than wild-type fruits. Antisense inhibition of *Nr*

receptor results in activation of ethylene responses and onset of normal ripening. Thus, the inability of the mutant *Nr* receptor to bind ethylene prevents its inactivation, and in *Nr* mutant fruit, ethylene responses are therefore suppressed (Hackett et al., 2000; Tieman et al., 2000). When *NR* expression is reduced by antisense technique, expression of *LeETR4* increased proportionately. In some manner, the plant compensates for reduced expression of *NR* by increasing expression of *LeETR4*. Thus, the overall receptor content of *NR* antisense lines is not substantially affected, whereas the receptor content in *LeETR4* antisense lines is substantially reduced.

Several groups have used the mutant ethylene receptor gene of *Arabidopsis etr1-1* to successfully control the ethylene perception. The initial evidence of the conserved role for the control of ethylene sensitivity was demonstrated using genetic transformation of the *Arabidopsis etr1-1* mutant gene into the heterologous species tomato and petunia (*Petunia* $\times$ *hybrida*). Using the constitutive CaMV 35S promoter to drive the expression of the dominant mutant *Arabidopsis etr1-1* gene, Wilkinson et al. (1997) were able to transform both species and obtain various ethylene-insensitive phenotypes. The wild-type tomato plants transformed with *etr1-1* displayed flower senescence and never ripe (NR) fruit phenotype, and *etr1-1* petunias produced flowers with delayed senescence after ethylene treatment and pollination. Further experiments utilizing a constitutive expression of the *Arabidopsis etr1-1* in petunias conferred ethylene insensitivity to the plants, but the constitutive expression of the gene had some additional negative effects (Clark et al., 1999; Gubrium et al., 2000; Clevenger et al., 2004). Even though the traits in the transformed petunias were dependent on the genetic background and temperature, their positive traits included delayed senescence and flower abscission, while the major negative trait was the poor rooting ability of the cuttings. Transgenic petunias with *etr1-1* under the control of floral-specific promoters of FBP1 (floral-binding protein) and AP3 (involved in floral development) genes had a vase life of up to five times that of nontransformed flowers (Cobb et al., 2002).

Bovy et al. (1999) found that expression of the *Arabidopsis etr1-1* gene in transgenic carnations under the control of either its own promoter (CMB2; carnation MADS box-containing promoter), the constitutive CaMV 35S, or the flower-specific petunia FBP1 promoter delayed flower senescence, resulting in a significant increase in vase life. Transgenic carnation cut flowers had three times the vase life of nontransformed flowers and lasted up to 16 days that was longer than flowers treated with either inhibitors of ethylene biosynthesis. Transgenic chrysanthemum "Sei-Marine" was generated by fusing the promoter of the tobacco elongation factor 1α (EF1α) gene to *DG-ERS1* cDNA and also by introducing one-nucleotide point mutations corresponding to those present in *Arabidopsis etr1-1*, *etr1-2*, *etr1-3*, and *etr1-4* and tomato *Nr* resulting in *mDG-ERS1(etr1-2)*, *mDG-ERS1(etr1-3)*, *mDG-ERS1(etr1-4)*, and *mDG-ERS1(Nr)* transgenes, respectively (Narumi et al., 2005b). Among the transgenes tested, the *mDG-ERS1 (etr1-4)* transgene showed a high ability to confer reduced ethylene sensitivity in chrysanthemum. This indicated the usefulness of the *mDG-ERS1* transgenes in conferring reduced ethylene sensitivity in chrysanthemum and gave further support for the action of the *DG-ERS1* gene in the perception of ethylene in chrysanthemum leaves.

Leaf and flower senescence were also delayed significantly in the transgenic coriander plants transformed with a mutated *Arabidopsis* ERS protein. The ability of the mutated *ERS1* gene to confer the ethylene-insensitive phenotype can be exploited in extending the shelf life of leafy vegetables (Wang and Kumar, 2004). Shaw et al. (2002) generated an

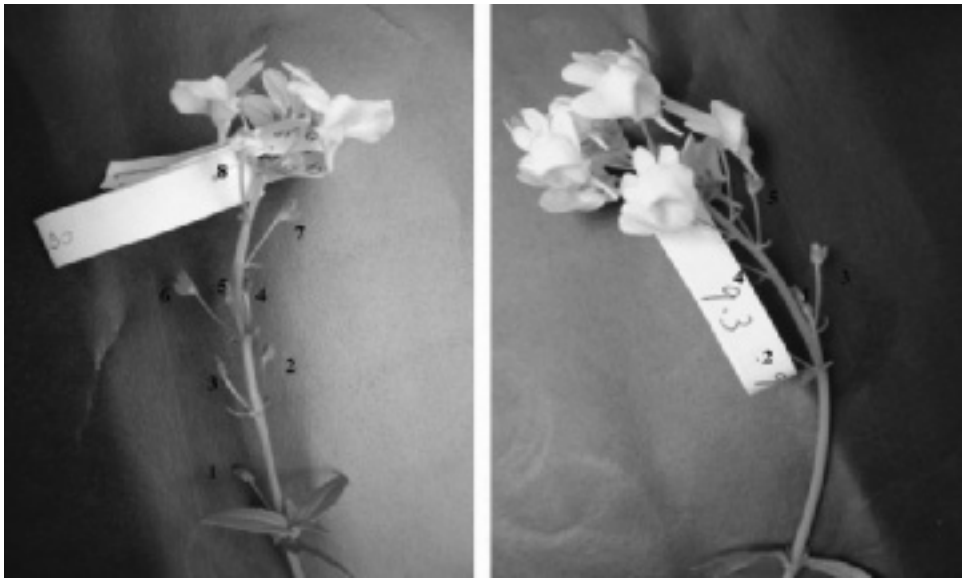

Fig. 6.4 Comparison of the flower longevity in *Nemesia*—wild type (left) and a transformant (right). The flower of the transgenic plant lasted longer than that of the wild-type plant; as a result, more flowers bloomed on the transgenic plant, simultaneously. The numbers indicate the flower positions in wild-type and transgenic *Nemesia* inflorescence.

ethylene-insensitive *Petunia* using a mutated *ers* homolog from *Brassica oleracea* (*boers*). Similar to *etr1-1*, *boers* codes for an ethylene receptor with a nonfunctional sensor domain that is not able to bind ethylene. The transgenic petunia plants were insensitive to ethylene, and produced flowers that were larger and had a longer vase life than those from nontransformed plants. However, the transformed plants had a higher mortality, due to a higher susceptibility to fungal diseases.

Recently, flower longevity in transgenic plants of an ethylene-sensitive ornamental plant, *Nemesia strumosa*, was established by introducing the mutated melon ethylene receptor gene *Cm-ETR1/H69A* (Cui et al., 2004; Takada et al., 2005). Based on the mutation in *Arabidopsis etr1-1*, the *mis*-sense mutation His-69 to Ala (H69A) was introduced into *Cm-ETR1* to create the mutant gene *Cm-ETR1/H69A*. The *Cm-ETR1/H69A* expression inhibited the ethylene response during the senescence of *Nemesia* flowers, resulting in longer shelf life (Fig. 6.4). This technique can be useful in delaying flower senescence in heterologous plants.

6.6 Conclusions

The case studies discussed in this chapter indicate that a substantial amount of research has been carried out on ethylene perception in fruits, vegetables, and flowers. The differences in ethylene response and/or differential gene expression observed during fruit ripening or flower senescence might reflect receptor function or interplay at these stages of development. The biological explanation and/or the significance of the multiplicity of ethylene receptors in plants are currently unknown, but it may be that individual receptors maintain a distinct

functional identity via the capacity to respond differentially to developmental and hormonal cues. The rationale and practical significance of studies on ethylene perception and gene expression is that if such a critical step in ethylene signal transduction can be understood, then, it would be possible to chemically or genetically modify plants in such a way as to prevent the increase in ethylene that leads to postharvest loss of plant quality. Nevertheless, the importance of receptor levels and possible receptor interplay during fruit ripening and flower senescence remain to be elucidated and is still a continuing area of study.

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Chapter 7

Enhancing Postharvest Shelf Life and Quality in Horticultural Commodities Using 1-MCP Technology

Susan Lurie and Gopinadhan Paliyath

7.1 Introduction

Ethylene is a plant hormone that regulates plant growth and developmental processes as well as ripening and senescence. The mode of action of ethylene thus may be influenced by the physiological status of the tissue. The production and continued action of ethylene are key factors that determine the shelf life and quality of harvested produce. Thus, several technologies have been developed with the objective of controlling these events. The use of the ethylene receptor blocker 1-methylcyclopropene (1-MCP) is one of the latest technologies that has entered the market. Application protocols for several commodities have been optimized and successfully employed worldwide.

1-MCP belongs to a class of cyclopropenes that were developed by Sisler and coworkers at the University of North Carolina. 1-MCP (Fig. 7.1) and the use of cyclopropenes for inhibiting ethylene action were patented by Sisler and Blankenship (1996). This was first tested as a gas on flowers, bananas, and tomatoes (Sisler et al., 1996; Sisler and Serek, 1997). A commercial breakthrough in 1-MCP application technology was the development of a stable formulation of 1-MCP as a powder in the form of a complex with cyclodextrin. The advantage of such a system was that 1-MCP could easily be released as a gas when the powder comes in contact with water. Development of improved chemicals and formulations is still underway; however, it is likely to remain as the primary means of controlling ethylene responses for several commodities in the immediate future (Sisler, 2006).

The impact of 1-MCP on postharvest science and technology has been twofold. First, it provides an efficient and simple technology to preserve fruit and vegetable quality after harvest. Second, 1-MCP has become a powerful tool to understand the fundamental mechanisms involved in ripening and senescence. Several reviews have recently been published on the effects of 1-MCP on horticultural commodities (Blankenship and Dole, 2003; Sisler and Serek, 2003; Watkins and Miller, 2005; Watkins, 2006). Updates on 1-MCP technologies can be obtained at http://www.hort.cornell.edu/mcp that catalogs the physiological and biochemical responses for each commodity.

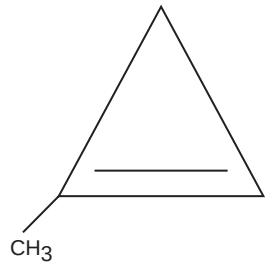

Fig. 7.1 Structure of 1-MCP.

7.2 Registration and application of 1-MCP

Commercial applications of 1-MCP were first made available for ornamental crops. This product with the trade name EthylBloc was approved by the United States Environmental Protection Agency (EPA) in 1999, and was marketed by Floralife, Inc. (Waterboro, SC). Further testing and registration of 1-MCP for edible crops were undertaken by AgroFresh Inc., a subsidiary of Rohm and Haas (Springhouse, PA). Several investigations have shown that 1-MCP is nontoxic to humans, has negligible residue, and is active at concentrations of parts per billion (ppb).

The safety, toxicity, and environmental profiles of 1-MCP to humans, animals, and in the environment were reported by the EPA (2002). The LC_{50} for inhalation in rats was greater than 2.5 mg/L (or 1,126 ppm active ingredient in air). Acute toxicity of 1-MCP or clinical signs of systemic toxicology have not been observed in toxicology studies (EPA, 2002).

With the approval by the EPA in 2002, 1-MCP is marketed under the trade name of SmartFresh. The use of SmartFresh has been approved by over 20 countries. The approval is for specific crops and includes apple, apricot, avocado, carrot, kiwifruit, mango, melon, nectarine, papaya, peach, pear, pepper, persimmon, pineapple, plantain, plum, squash, tomato, and tulip bulbs. Table 7.1 gives a summary of the fruits and vegetables that respond to 1-MCP.

At 20°C, 1-MCP is released as a gas from a formulated cyclodextrin powder when it is mixed with water in nearly 20–30 min. A complete release may take longer duration at lower temperatures. Under such conditions, 1-MCP is absorbed by the harvested material over time. Peaches absorbed 80–90% of the 1 μL/L applied either at 20°C or 0°C when held in a closed container for 20 h (Liguori et al., 2004). As well, an increase in CO_2 levels due to respiration does not inhibit the effectiveness of binding of 1-MCP (Blankenship and Dole, 2003) in closed systems. In addition, ethylene is also produced by harvested materials. Since 1-MCP shows a higher affinity toward the ethylene receptor, nearly 100-fold higher levels of ethylene are required to effectively compete for the binding site. The highest concentration of 1-MCP that is approved for use is 1 μL/L. After binding to the receptors, 1-MCP does not get released, and any reversal of 1-MCP treatment is potentially because of the synthesis of new ethylene receptors in the tissue (E.C. Sisler, personal communication). Repeated weekly applications of 1-MCP (Mir et al., 2001; Mir and Beaudry, 2001; Jayanty et al., 2004) helped prevent apple softening at 20°C more than at 0°C, and this was attributed to an increased turnover of the receptor at high storage temperature.

It has also been noticed that 1-MCP can be absorbed or adsorbed nonspecifically to sites other than ethylene receptors. In storage rooms, various plastics did not absorb the

Table 7.1 Climacteric, nonclimacteric fruit, and vegetables for which responses to 1-MCP have been investigated, and the range of 1-MCP concentration utilized

	Range of 1-MCP concentrations (μL/L)
Climacteric fruit	
Apple (*Malus sylvestris* (L.) Mill. var. *domestica* (Borkh.) Mansf.)	0.01–1
Apricot (*Prunus armeniaca* L.)	0.3–1
Avocado (*Persea americana* Mill.)	0.03–1
Banana (*Musa* L.)	0.01–1
Blueberry (*Vaccinium corymbosum* L.)	0.1–0.4
Chinese bayberry (*Myrica rubra* Siebold and Zuccarni)	1–10
Custard apple (*Annona squamosa* L.)	25
Fig (*Ficus carica* L.)	0.4–2.5
Guava (*Psidium guajava* L.)	0.1–0.9
Kiwifruit (*Actinidia deliciosa*)	0.5–5
Jujube, Chinese (*Zizyphus jujube*) and Indian (*Zizyphus mauritina*)	0.6
Loquat (*Eriobotrya japonica* Lindl.)	0.5–50
Lychee (*Lichi chinensis*)	1
Mamey sapote (*Pouteria sapote* (Jacq.))	1
Mango (*Mangifera indica* L.)	25–100
Melon (*Cucumis melo* L.)	1
Mountain papaya (*Vasconcellea pubescens*)	0.3
Nectarine (*Prunus persica* Lindl.)	0.25–5
Papaya (*Carica papaya* L.)	0.05–2.5
Peach (*Prunus persica* L. Batsch)	0.25–5
Pear (*Pyrus communis* L.)	0.01–1
Pear, Asian (*Pyrus pyrifolia* Nakai)	0.1–1
Persimmon (*Diospyros khaki* L.)	0.01–3
Plum (*Prunus salicina* Lindl.)	0.1–2
Tomato (*Lycospersicum esculentum* Mill.)	0.1–1
Nonclimacteric fruit	
Cherry (*Prunus avium* L.)	0.2–0.4
Clementine mandarin (*Citrus reticulata* L.)	0.1–1
Cucumber (*Cucumis sativus* L.)	1–10
Grape (*Vitis vinefera* L.)	4
Grapefruit (*Citrus paradisi* Macf.)	1
Lime (*Citrus latifolia* Tanaka)	0.25–1
Orange (*Citrus sinensis* L. Osbeck)	0.1–5
Pepper (*Capsicum frutenscens* L.)	0.25–1
Pineapple (*Ananas comosus* L.)	0.1
Pomegranate (*Punica granatum)*	1
Strawberry (*Fragaria* × *ananassa* Duch.)	0.01–1
Watermelon (*Citrullus lanatus*)	0.5–1
Vegetables	
Broccoli (*Brassica oleracea* L.)	0.02–50
Carrot (*Daucus carota* L.)	42
Chayote (*Sechium edule* Jacq.)	0.3–1.2
Chinese cabbage (*Brassica campestris* L.)	1
Chinese mustard (*Brassica juncea* var. *foliosa*)	1
Choy sum (*Brassica rapa* var. *parachinensis*)	1
Coriander (*Coriandrum sativum* L.)	0.1–10
Lettuce (*Lactuca sativa* L.)	0.5–1
Mibuna and mizuna (*Brassica rapa* var. *nipposinica*)	1
Mint (*Mentha longifolia* L.)	0.5

(continued)

Table 7.1 (*Continued*)

	Range of 1-MCP concentrations (μL/L)
Onion (*Allium cepa* L.)	1
Pak choy (*Brassica rapa*)	1
Parsley (*Petroselinum crispum* Mill.)	10
Potato (*Solanum tuberosum*)	0.55–2.64
Rocket (*Eruca sativa* Mill.)	0.5
Tatsoi (*Brassica rapa* var. *rosularis*)	1

compound; however, wood or cardboard materials absorbed 1-MCP. Moistened wood absorbed the compound at a higher rate. Thus, it appears that 1-MCP levels may be compromised by wooden and cardboard bins and bin liner materials, but not by plastic bin or wall surface materials (Vallejo and Beaudry, 2006). This also suggests that 1-MCP is readily absorbed to cellulosic materials. One study suggests that boxed fruit absorbed 1-MCP better compared to bulk stored fruit, provided that the boxes were well ventilated (Valero et al., 2004).

In a study comparing the absorption of 1-MCP by fruits, a faster decline of 1-MCP was observed in a container with avocados than apples (Dauny et al., 2003). A higher oil content in avocados may have enhanced the absorption of 1-MCP. A recent study (Nanthachai et al., 2007) has examined the ability of a number of commodities to absorb 1-MCP including apple, asparagus, ginger, green bean, key lime, lettuce, mango, melon, parsnip, plantain, potato, and tangerine. The rate of absorption varied 30-fold among different commodities tested. The dry matter content and the size of commodity influenced the rate of absorption.

AgroFresh is in the process of optimizing a formulation of 1-MCP that can be used as a liquid spray in the field or orchard before harvest, and its effect has recently been reported (Watkins et al., 2006). As well, a sachet release system that would supply controlled release of 1-MCP into a package containing fresh produce (Lee et al., 2006) is also under development. To obtain a sustained release during the shipment of flowers, SmartFresh was included in polyvinylchloride tubes (Macnish et al., 2004). Such advances will enable targeted use of the product to obtain more efficient shelf life and quality preservation in commodities.

7.3 Responses of climacteric fruit

The following are specific effects observed with 1-MCP treatment in several climacteric fruits.

7.3.1 Apple

1-MCP application delays the increase in ethylene production and levels of internal ethylene in apple fruits (Fig. 7.2). It is most effective at concentrations in the range of 0.5 to 1 μL/L. As well, the response may depend on the type of cultivar, storage conditions, and the duration of storage (Fan et al., 1999; Rupasinghe et al., 2000; Watkins et al., 2000; Dauny and Joyce,

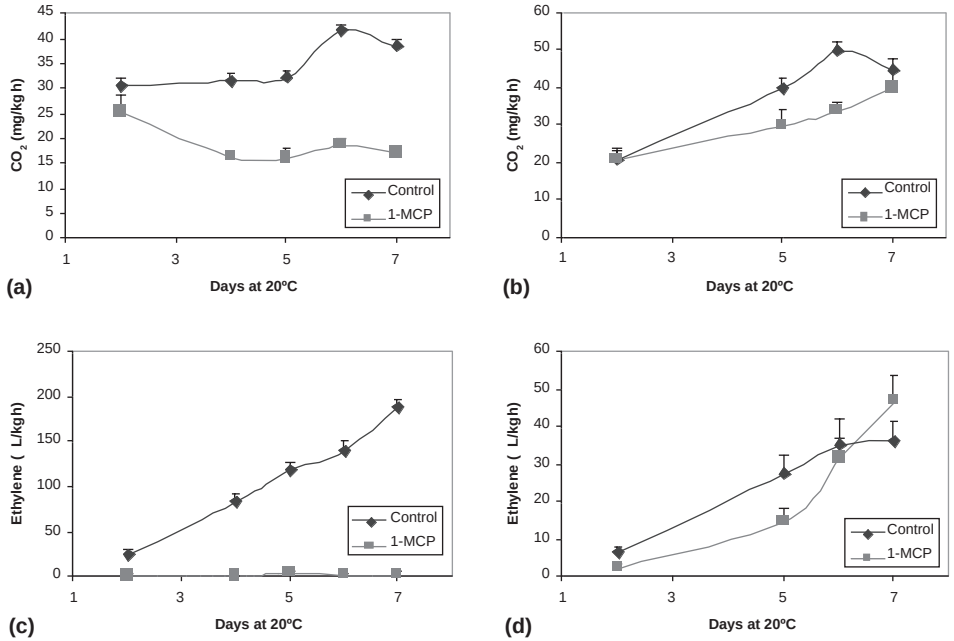

Fig. 7.2 The differing responses of apple and nectarine ethylene production and respiration to 1-MCP. (a) and (c) Respiration and ethylene production of "Anna" apples, and (b) and (d) respiration and ethylene production of "April Glow" nectarines.

2002; Jiang and Joyce, 2002; Pre-Aymard et al., 2003; Saftner et al., 2003; Mattheis et al., 2005; Moran and McManus, 2005; Watkins and Nock, 2005). Respiration is also inhibited by 1-MCP to a lesser extent than ethylene inhibition (Fig. 7.2).

A dramatic effect of 1-MCP treatment is the prevention/delay of softening (Fan et al., 1999; Rupasinghe et al., 2000; Watkins et al., 2000; Mir et al., 2001; Dauny and Joyce, 2002; Pre-Aymard et al., 2003; Saftner et al., 2003; Zanella, 2003; Defilippi et al., 2004; Bai et al., 2005; Mattheis et al., 2005; Moran and McManus, 2005). The fruit texture is also affected with increased crispiness (Baritelle et al., 2001; Pre-Aymard et al., 2005). In contrast to cold storage after treatment with 1-MCP, storage at ambient temperature helps retain the firmness better (Fan et al., 1999; Mir et al., 2001). Toivonen and Lu (2005) reported that 1-MCP did not affect firmness of an early-ripening summer apple when the fruits were stored below 15°C. However, another summer apple, "Anna," responded well to 1-MCP when stored at 0°C (Pre-Aymard et al., 2003). In some cultivars, loss of green color due to ripening is prevented by 1-MCP treatment (Pre-Aymard et al., 2003; Saftner et al., 2003; Zanella, 2003).

In general, 1-MCP treatment delays loss of titratable acidity (TA), but does not affect soluble solids content (SSC) in any consistent pattern (Pre-Aymard et al., 2003, 2005; Saftner et al., 2003; Zanella, 2003; Defilippi et al., 2004; Bai et al., 2005; Moran and McManus, 2005; Toivonen and Lu, 2005). However, mixed responses depending on whether the fruit were held in controlled atmosphere (CA) or in air have been reported (Watkins et al., 2000). The evolution of aroma volatiles is reduced by 1-MCP treatment, although individual volatiles may be affected differentially (Rupasinghe et al., 2000; Lurie et al.,

2002; Saftner et al., 2003; Defilippi et al., 2004; Bai et al., 2005; Kondo et al., 2005). During ripening, the aliphatic alcohols are used for the biosynthesis of esters, and this process is inhibited by 1-MCP. Ester volatile biosynthesis is mediated by alcohol acyl CoA transferase (AAT). The expression and activity of apple alcohol transferase gene (*MdAAT2*) was inhibited in 1-MCP-treated apples during storage (Li et al., 2006). In response to 1-MCP treatment, the rapidly ripening summer apple "Anna" showed less fruity, ripe, and overall aromas which resembled that from a less ripe apple (Lurie et al., 2002). This was due to an inhibition of volatile ester biosynthesis and maintenance of alcohol levels in the treated fruit.

Improvement or preservation of nutritional quality is a key aspect of any postharvest technology. There are conflicting reports on the effect of 1-MCP on apple antioxidants. In Granny Smith (Shaham et al., 2003) and Golden Smoothee apples (Vilaplana et al., 2006), total antioxidant activity and ascorbate levels were not affected by 1-MCP. 1-MCP-treated apples were under lower oxidative stress, having lower levels of hydrogen peroxide and peroxidative markers. In another study, the total oxyradical scavenging capacity of both "Delicious" and "Empire" apples was higher in storage when treated with 1-MCP (MacLean et al., 2003). MacLean et al. (2006) also found that the flavonoid content of the apple fruit was slightly higher in 1-MCP-treated fruit, and the anthocyanin levels were preserved during storage. However, chlorogenic acid, a major apple flavonoid, was 24% lower in 1-MCP-treated apples. Shaham et al. (2003) found no difference in flavonoid levels between control and 1-MCP-treated apples during storage.

Ethylene production by apple fruit occurs both on and off the tree when the ripening is initiated. Therefore, the effectiveness of 1-MCP application is influenced by the maturity/ripening stage at harvest and by the period the fruits are held in cold storage before treatment. These two factors are interrelated, as more mature fruit at the time of harvest produce autocatalytic ethylene sooner than earlier harvested less mature fruit. The effect of any delays between harvest and application of 1-MCP is influenced by the cultivar as well as the type and length of storage (Mir et al., 2001; Watkins and Nock, 2005). In a comparative study of two apple cultivars "Orin" and "Fuji" that show differences in ethylene production, ethylene was inhibited by 1-MCP in "Fuji" apples even when treatment was delayed for a week after storage (Tatsuki et al., 2007). The expression levels of two ethylene receptor genes, *MdERS1* and *MdERS2*, and the ACC-synthase gene *MdACS1* were also inhibited in "Fuji" apples by 1-MCP, while ACC-oxidase *MdACO1* was inhibited slightly. However, in a high ethylene-producing apple, "Orin," the later the 1-MCP application after harvest, the lesser was the suppression of ethylene production and the expression of these genes. In a comparison of apple peel and pulp and the responses of the ethylene biosynthetic pathway to 1-MCP, it was found that ACC synthase and ACC levels were decreased in both peel and pulp (Vilaplana et al., 2007). ACC oxidase was also inhibited but not totally. However, high levels of malonyl ACC were found in 1-MCP-treated tissue.

Controlled atmosphere storage can prolong the effect of 1-MCP on both physical and sensory qualities of apple fruit (Rupasinghe et al., 2000; Watkins et al., 2000). However, 1-MCP treatment and short-term air storage may replace the requirement for CA storage, especially for maintaining quality of summer apples (Pre-Aymard et al., 2003), and of those cultivars that show enhanced deterioration after 2–3 months of storage (Watkins et al., 2000; Dauny and Joyce, 2002; Bai et al., 2005). In addition, 1-MCP treatment can be effective in many places where CA rooms are not available.

7.3.2 Avocado

Unlike apples, avocados do not ripen on the tree, and the fruits begin the ripening process after they are harvested. Avocados respond to 1-MCP in a concentration and time-dependent manner (Feng et al., 2000; Jeong et al., 2002). An earlier study used excessive levels of 1-MCP (25 μL/L) (Hofman et al., 2001), however, because of its sensitive nature concentrations above 0.25 μL/L inhibits ripening (Jeong et al., 2003; Adkins et al., 2005; Woolf et al., 2005). The inhibition of ripening is associated with a delay as well as a decrease in ethylene production and the respiratory climacteric (Feng et al., 2000, 2004; Jeong et al., 2002, 2003; Hershkovitz et al., 2005). The treated fruits show increased firmness, delayed softening, and change in skin color. 1-MCP also reduces the weight loss during storage (Jeong et al., 2003).

The activities of enzymes involved in cell wall disassembly have also been evaluated after subjecting avocado fruits to 1-MCP treatment (Feng et al., 2000; Jeong and Huber, 2005). Inhibition of both endo-β-1,4-glucanase and polygalacturonase activities was associated with retention of firmness in avocado fruits. However, complete suppression of polygalacturonase and inhibition of endo-β-1,4-glucanase and β-galactosidase were observed in the later study (Jeong and Huber, 2005). However, even in the absence of any measurable activity of polygalacturonase, 1-MCP-treated fruits lost nearly 80% of their initial firmness after 24 days of storage at 20°C.

In the case of avocados, it is important that the 1-MCP treatment procedure does not delay the ripening procedure excessively. A long delay in ripening is also associated with an increase in fruit decay (Adkins et al., 2005; Wang et al., 2006). However, 1-MCP reduces ethylene-induced storage disorders in storage (Pesis et al., 2002; Woolf et al., 2005), making this an alternative approach for disease control.

7.3.3 Banana

Bananas are harvested at a mature green stage of maturity and ripened by the application of ethylene. 1-MCP treatment of the fruits delayed ripening in a time- and concentration-dependent manner (Jiang et al., 1999b; Harris et al., 2000; Bagnato et al., 2003). The inhibition by 1-MCP was also dependent on the maturation stage. The ripening of immature bananas is inhibited to a lesser extent than that of mature fruits by 1-MCP (Harris et al., 2000). As well, once ripening is initiated by propylene treatment of mature green bananas, 1-MCP treatment does not inhibit the ripening processes effectively (Golding et al., 1998).

As in other fruits, a decrease in ethylene production and respiration rates and an inhibition of softening were evident in bananas exposed to 1-MCP (Golding et al., 1998; Jiang et al., 1999a, b; Macnish et al., 2000; Pathak et al., 2003; Pelayo et al., 2003; Lohani et al., 2004). A lower level of soluble solids was detected in a study (Nascimento et al., 2006). As in apples (Lurie et al., 2002), total volatile production was decreased and ester concentrations were lower, while those of alcohols were higher in treated fruit (Golding et al., 1998). A number of ripening-related genes were upregulated by ethylene treatment in banana, and this increase was prevented by 1-MCP (Gupta et al., 2006). Two genes for enzymes induced by ethylene are a fruit-specific expansin, *MaExp1*, and one for β-amylase. These enzymes degrade starch into sugars, and both were inhibited by 1-MCP treatment (Trivedi and Nath, 2004; Nascimento et al., 2006). An ethylene-responsive, ripening-related expansin gene,

MiExpA1, has also been found in mango, and its expression was also inhibited by 1-MCP (Sane et al., 2005).

The change in skin color of bananas from green to yellow indicative of chlorophyll degradation was inhibited by 1-MCP. However, the color changes during ripening of 1-MCP-treated fruits are not uniform, having disrupted or incomplete and uneven yellowing (Golding et al., 1998; Harris et al., 2000; Macnish et al., 2000). This may be a limitation for the use of 1-MCP on bananas. As well, application of 1-MCP after an ethylene treatment of fruits was unsuccessful in circumventing this problem (Pelayo et al., 2003). As well, due to the physiological variation even within a bunch, there was considerable variation in responses of the fruit. In another study using propylene to induce ripening, application of 1-MCP 24 h after propylene treatment inhibited both color and volatile production, but not ethylene or respiration (Golding et al., 1998).

7.3.4 Pear

1-MCP has been tested for its effectiveness on summer and winter pears. Pear fruit requires an exposure to chilling temperatures before they begin to ripen. Winter pears require as much as 8 weeks at low temperature before they ripen. Winter pears soften and develop a buttery texture, while summer pears better retain their crispiness after they ripen. 1-MCP treatment delayed or prevented softening, the degree of response depended on the cultivar and the concentration of 1-MCP applied (Baritelle et al., 2001; Argenta et al., 2003; Hiwasa et al., 2003; Kubo et al., 2003; Calvo and Sozzi, 2004; Ekman et al., 2004; Trinchero et al., 2004). An effective concentration that can delay ripening without preventing the process was 0.2 μL/L (Calvo and Sozzi, 2004; Moya-Leon et al., 2006). As in other green fruits, the change in peel color from green to yellow was inhibited as well as ethylene production and respiration (Argenta et al., 2003; Hiwasa et al., 2003; Kubo et al., 2003; Ekman et al., 2004; Larrigaudiere et al., 2004; Trinchero et al., 2004; Mwaniki et al., 2005). The soluble solids content was not affected in pears after 1-MCP treatment, while changes in total acidity were inconsistent (Argenta et al., 2003; Calvo and Sozzi, 2004; Larrigaudiere et al., 2004; Trinchero et al., 2004). In a sensory evaluation on "Packham's Triumph" pears, the flavor and aroma profile of 1-MCP-treated fruit stored in air were preferred over CA-stored fruit (Moya-Leon et al., 2006).

There is variability in concentrations of 1-MCP that delay but do not prevent pear ripening. Application of 0.2 μL/L resulted in normal ripening with no overripening (Calvo and Sozzi, 2004; Moya-Leon et al., 2006), while concentrations as high as 10 μL/L resulted in maintenance of optimal eating firmness for extended periods (Kubo et al., 2003). The efficiency of ethylene to reverse 1-MCP effects was dependent on the concentration of 1-MCP application and the length of time the fruit had been stored (Argenta et al., 2003; Calvo and Sozzi, 2004; Ekman et al., 2004).

7.3.5 Peach and nectarine

Responses of fruit to 1-MCP are affected by concentration and exposure period, but are not dependent on treatment temperature (Liguori et al., 2004). Inhibition of fruit ripening by 1-MCP was not persistent; however, repeated applications of 1-MCP helped maintain suppression of ripening (Liu et al., 2005). The transitory effect of 1-MCP was not due to any

diffusion limitations within the flesh (Hayama et al., 2005). It is probable that the ethylene receptors are rapidly turned over, which help overcome the 1-MCP effect in the fruit tissue (Dal Cin et al., 2006). This turnover was not found in apples, which may help explain the prolonged effect of 1-MCP on apple fruit (Dal Cin et al., 2006).

Ethylene production can be variable in 1-MCP-treated peaches and nectarines (Mathooko et al., 2001; Fan et al., 2002; Rasori et al., 2002; Liguori et al., 2004; Bregoli et al., 2005; Girardi et al., 2005). 1-MCP-treated fruits showed a higher level of ethylene production than untreated fruits (Rasori et al., 2002), and the transcript levels of ACS and ACO were higher in treated fruit (Bregoli et al., 2005). The respiration rates are also variable after 1-MCP treatment (Fig. 7.2) (Dong et al., 2001b; Fan et al., 2002; Liguori et al., 2004). Softening is delayed in 1-MCP-treated fruits, but this effect is not retained during further storage (Dong et al., 2001b; Mathooko et al., 2001; Liguori et al., 2004). The effect on soluble solids content is also variable; however, decline in total acidity is slowed in high acid (Fan et al., 2002; Liguori et al., 2004; Bregoli et al., 2005; Liu et al., 2005), but not in low-acid cultivars.

7.3.6 Plum

Among stone fruits, plums are much more sensitive to 1-MCP than peaches and nectarines. Japanese-type plums show either climacteric or suppressed climacteric character. The climacteric rise in respiration and increase in ethylene production are temporally delayed in suppressed climacteric fruit during ripening. Ripening of suppressed climacteric cultivars was inhibited when treated with 1 μL/L 1-MCP unless followed by a subsequent treatment with propylene (Abdi et al., 1998). When treated with 0.1 μL/L 1-MCP, the fruit eventually ripened and softened (Dong et al., 2001a).

1-MCP treatment prevented or delayed the climacteric increase in ethylene production and respiration of plums (Dong et al., 2002; Martinez-Romero et al., 2003; Salvador et al., 2003; Valero et al., 2003, 2004; Khan and Singh, 2007). In addition, softening and skin color changes were delayed and weight loss was reduced (Dong et al., 2001a, 2002; Menniti et al., 2004; Khan and Singh, 2007). Increase in soluble solids content was also affected (Valero et al., 2004).

Postharvest softening and susceptibility to mechanical injury and pathogens are major problems that limit shipping and shelf life of plums. The response of plums to 1-MCP is variable based on cultivar and harvest maturity, but there are reports of large extension of storage and shelf life of treated fruit due to a decrease in mechanical damage and decay (Abdi et al., 1998; Martinez-Romero et al., 2003; Khan and Singh, 2007). Moreover, 1-MCP is more effective in mature fruit showing better organoleptic quality (Salvador et al., 2003; Valero et al., 2003).

7.3.7 Tomato

Tomato was one of the earliest fruits examined for the effectiveness of 1-MCP by Sisler et al. (1996). 1-MCP treatment inhibited ethylene production and respiration, fruit softening, color changes, and decrease in total acidity, but did not change the soluble solids content. The extent of ripening inhibition in tomato fruit is influenced by 1-MCP concentration, time of exposure, maturity stage, and cultivar types (Sisler et al., 1996; Hoeberichts et al., 2002;

Wills and Ku, 2002; Mir et al., 2004; Opiyo and Ying, 2005; Guillen et al., 2006, 2007). Fruits regain the capacity to ripen after treatment, but ripening can be delayed by a second application (Hoeberichts et al., 2002; Mir et al., 2004). Fruit treated at pink and light red stages ripened properly after a delay (Hurr et al., 2005), while red ripe fruit had a longer shelf life of only 1 day when treated with 1-MCP (Ergun et al., 2006a).

Marketing of tomatoes in bunches with the tomatoes still attached to the stem is becoming more popular. 1-MCP has been found to inhibit the abscission of cherry tomatoes from the vines (Beno-Moualem et al., 2004; Lichter et al., 2006). The concentration of 1-MCP required to inhibit fruit abscission may be much higher, and this may negatively affect the ripening pattern. In citrus plantations, ethephon sprays are conducted to enhance fruit loosening, with an undesired side effect of leaf abscission. 1-MCP was found to inhibit leaf drop without affecting the ability of ethephon to cause fruit loosening (Pozo et al., 2004).

7.3.8 Other climacteric fruit

Tropical fruits, in general, are fast ripening and possess very low shelf life. Tropical fruits are also susceptible to chilling-injury development, and therefore, 1-MCP may be of great potential benefit. As well, cold storage or controlled atmosphere facilities are lacking in many tropical countries, and fruits are stored at ambient temperature. Guava, mamey sapote, and mountain papaya are three exotics that have been tested with 1-MCP. Guava responded to 1-MCP treatment with a twofold increase in shelf life, although at a high dosage (0.9 μL/L for 6 h or longer), the fruit failed to ripen (Bassetto et al., 2005). A similar effect was observed in other fruits such as kiwifruit (Boquete et al., 2004) and papaya (Manenoi et al., 2007), while some fruits, such as stone fruits did not appear to have an upper limit of 1-MCP. Mamey sapote is a large fruit grown in Central America and the Caribbean that softens rapidly after harvest. 1-MCP increased the shelf life of this fruit and also retained total acidity without affecting soluble solids content (Ergun et al., 2005). Mountain papaya ripens by rapid degreening followed by increase in respiration, ethylene production, and flesh softening. 1-MCP prevented the increase in ethylene and partially inhibited softening and color development in this fruit (Moya-Leon et al., 2004). The ripening of papaya is associated with a strong and characteristic aroma due to increased production of esters and alcohols (Balbontin et al., 2007). Ethylene (as ethrel) promotes and 1-MCP inhibits the aroma component production in papaya.

Persimmon fruits treated with 1-MCP showed slower ripening and softening after reduction in astringency (Harima et al., 2003; Salvador et al., 2004a; Luo, 2007). Cultivars that are susceptible to chilling injury also had less internal gel formation, possibly because of inhibited softening (Salvador et al., 2004b).

7.4 Physiological storage disorders

The effect of 1-MCP has been thoroughly investigated in reducing physiological disorders of apples that occur during storage. Several studies were in relation to superficial scald, since an interaction between ethylene production and α-farnesene was believed to be a primary cause of scald development, and early reports indicated that 1-MCP inhibited superficial scald development (Fig. 7.3) (Fan and Mattheis, 1999a, b; Rupasinghe et al., 2000; Watkins et al., 2000). Superficial scald appears as a brown discoloration on the

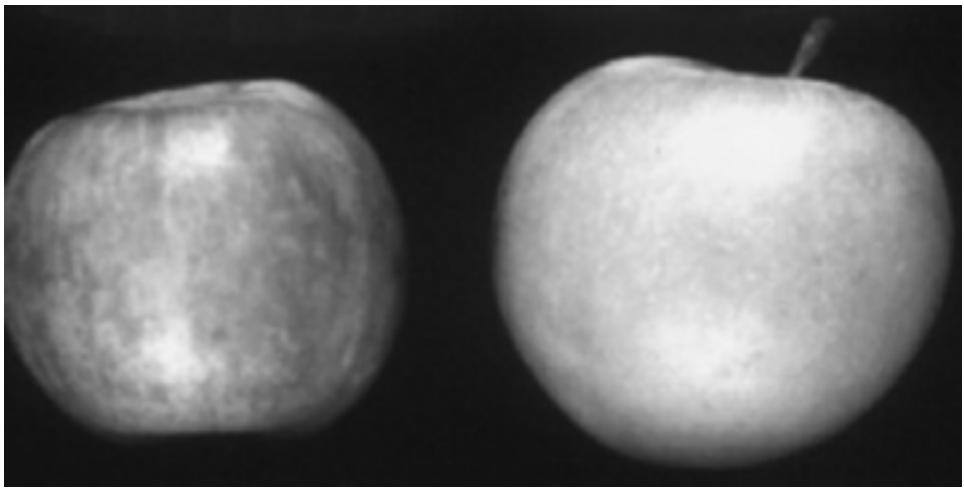

Fig. 7.3 Superficial scald on "Granny Smith" apples and its prevention by 1-MCP treatment at harvest. The apples were stored for 5 months at 0°C and held 5 days at 20°C.

peel in apple and as black patches on pear. Superficial scald has also been termed as a chilling-injury symptom as it occurs during low-temperature storage (Watkins et al., 1995). Mechanistically, the accumulation of α-farnesene and its oxidation to conjugated trienols in the peel region and associated cell damage is believed to cause the development of this symptom (Rowan et al., 1995; Whitaker et al., 1997). α-Farnesene production is enhanced by ethylene (Du and Bramlage, 1994; Watkins et al., 1995; Whitaker et al., 2000). Superficial scald can be prevented or alleviated by inhibiting α-farnesene production or its oxidation. It has been demonstrated that inhibition of scald by 1-MCP is associated with inhibition of α-farnesene accumulation, and therefore, less substrate for oxidation (Fan and Mattheis, 1999a; Rupasinghe et al., 2000; Watkins et al., 2000; Shaham et al., 2003; Arquiza et al., 2005; Pechous et al., 2005).

Other apple disorders have also been found to be affected by inhibition of ethylene signaling. 1-MCP can reduce senescent breakdown (Watkins et al., 2000; DeLong et al., 2004; Moran and McManus, 2005), core flush or brown core (Fan and Mattheis, 1999a; Zanella, 2003; DeLong et al., 2004), and soft scald (Fan and Mattheis, 1999a). These disorders are associated with senescence and cold storage. The development of greasiness in some apple cultivars, such as "Granny Smith," is also a process that develops in cold storage, and this is inhibited by 1-MCP (Fan and Mattheis, 1999a; Watkins and Nock, 2005).

One problem that has been found as 1-MCP is used commercially is carbon dioxide injury that is higher in 1-MCP-treated fruit than untreated fruit (Zanella, 2003; Watkins and Nock, 2005). This may be related to the application of early CA to less mature fruits from early harvests (Watkins et al., 1997; Fernandez-Trujillo et al., 2001). Since 1-MCP delays the ripening process, MCP treatment may enhance the injury. The disorder can be alleviated by maintaining low carbon dioxide in the storage room for the first few weeks (Watkins and Nock, 2005). Alternatively, since 1-MCP mimics the beneficial effect of CO_2 on firmness retention, CO_2 could be eliminated or reduced in CA regimes for "Empire" apples treated with 1-MCP (DeEll et al., 2005).

Superficial scald in pears is also inhibited by 1-MCP, although it may appear as the MCP effect wears off (Ekman et al., 2004). 1-MCP inhibits the accumulation of α-farnesene and conjugated trienols in pears (Isidoro and Almeida, 2006). The inhibition appeared to be at the level of gene transcription since the expression of *PcAFS1* was reduced in 1-MCP-treated fruit (Gapper et al., 2006). Other disorders in pears, similar to those in apples, are also alleviated by 1-MCP. These include senescent scald and core browning (Argenta et al., 2003), internal and senescent breakdown (Kubo et al., 2003; Ekman et al., 2004), and watery and core breakdown (Calvo and Sozzi, 2004). The development of scratches or browning on the peel is also delayed by 1-MCP.

Other low-temperature disorders on a number of fruits can be inhibited by 1-MCP. These include internal flesh browning in avocado (Pesis et al., 2002; Hershkovitz et al., 2005; Woolf et al., 2005), loquat (Cai et al., 2006a), pineapple (Selvarajah et al., 2001), and chilling injury of citrus fruit (Dou et al., 2005). However, an early study reported that chilling injury in citrus was enhanced by 1-MCP (Porat et al., 1999). Scald on pomegranate can be reduced but not eliminated by 1-MCP (Defilippi et al., 2006). This scald in not due to α-farnesene oxidation, but it correlated with phenol levels in the peel (Ben-Arie and Or, 1986). Reduced browning is associated with reduced polyphenol oxidase and peroxidase activities (Pesis et al., 2002; Hershkovitz et al., 2005). Biosynthesis of isocoumarins in carrots, which leads to bitterness development, is inhibited by 1-MCP treatment (Fan et al., 2000; Fan and Mattheis, 2001). As well, the increase in fruit firmness during low-temperature storage due to tissue lignification in loquat fruit was mitigated by 1-MCP (Cai et al., 2006b). In watermelon, ethylene treatment causes water soaking due to an increase in the activity of lipid-degrading enzymes and phospholipid degradation (Mao et al., 2004). This disorder is prevented by prior exposure to 1-MCP.

In stone fruits such as peaches and nectarines, the development of chilling-injury symptoms such as internal browning, flesh wooliness, and red color (Fig. 7.4) was increased by 1-MCP (Dong et al., 2001b; Fan et al., 2002; Girardi et al., 2005). In these fruits, a certain level of ethylene production is necessary for normal ripening to occur after storage (Dong et al., 2001b; Zhou et al., 2001). In apricots, 1-MCP enhanced internal browning even without storage (Dong et al., 2002). In plums, however, 1-MCP has been found to decrease internal browning during storage (Menniti et al., 2006). Also, impact injury in apricots (De Martino et al., 2006) and in European plums (Lippert and Blank, 2004) was decreased by 1-MCP, due to its inhibition of softening.

7.5 Responses to pathogens

The effects of 1-MCP in prevention of pathological disorders that occur during storage have not been investigated in depth. Because of its involvement in regulation of plant defense genes, low levels of endogenous ethylene may be required to maintain basic levels of resistance.

In two nonclimacteric fruits, citrus and strawberry, 1-MCP has been reported to enhance decay. In citrus, 1-MCP treatment effectively inhibited the ethylene effect on the degreening process, but it increased mold rots caused by *Penicillium digitatum* and *Penicillium italicum*, and stem-end decay caused by *Diplodia natalensis* (Porat et al., 1999; Marcos et al., 2005). However, in grapefruit inoculated with *P. digitatum*, 1-MCP did not affect the process of decay development (Mullins et al., 2000).

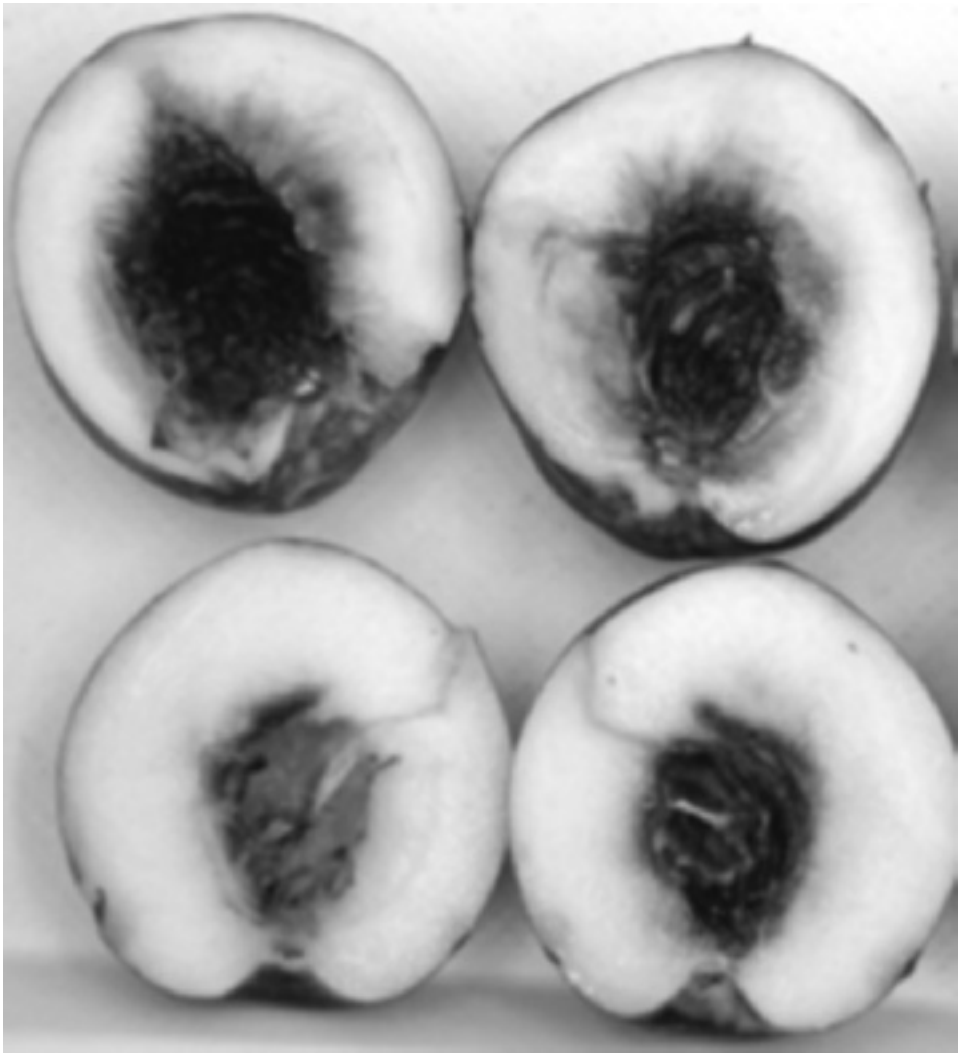

Fig. 7.4 Internal reddening of peaches treated with 1-MCP at harvest, stored 3 weeks at 0°C, and then 5 days at 20°C. (Top) 1-MCP treated; (bottom) control.

Strawberry fruits exposed to 1-MCP retained firmness and color better. However, disease development was accelerated in fruit treated at high (0.5 and 1 μL/L) 1-MCP concentrations, though not at lower concentrations (Ku et al., 1999b; Jiang et al., 2001). In another study, exposure of strawberries to 0.01, 0.1, or 1 μL/L 1-MCP did not affect overall fruit acceptability, but did slightly increase the rate of rot development (Bower et al., 2003).

Apples were found to be more susceptible to bitter rot (*Colletotrichum acutatum*) and blue mold (*Penicillium expansum*) in 1-MCP treated than untreated fruit (Janisiewicz et al., 2003). However, Saftner et al. (2003) found that 1-MCP reduced decay when fruits were inoculated at harvest with *P. expansum*, *Botrytis cinerea*, and *C. acutatum* and subsequently stored in controlled atmosphere. It appears that increased firmness due to 1-MCP treatment

enhanced the resistance to pathogen infection. In pears, stem-end decay was reduced after inoculating the fruit with *B. cinerea* and treating with 0.1 μL/L 1-MCP than without treatment (Spotts et al., 2007). Natural infections caused by *Phacidiopycnis piri* was also reduced in 1-MCP-treated fruit, and the fruit remained firmer in storage than untreated fruit.

1-MCP increased disease susceptibility of custard apple, mango, papaya (Hofman et al., 2001), and avocado (Hofman et al., 2001; Adkins et al., 2005; Woolf et al., 2005). Avocado has an antifungal diene compound that is present in unripe fruit and its level is enhanced by ethylene (Leikin-Frenkel and Prusky, 1998). Latent infections of *C. gloeosporioides* were inhibited from developing until the level of the diene compound declined during fruit ripening. 1-MCP treatment of avocado at two stages of maturity prevented further diene synthesis, but the early-harvested fruit had high initial levels of diene and because of the inhibitory effect on ripening, 1-MCP decreased decay development. In the later harvest, the level of diene was lower and decay developed more rapidly in treated fruit than untreated fruit (Wang et al., 2006).

Most studies on stone fruits found benefits of 1-MCP treatment in reducing decay. In two plum cultivars, "Fortune" and "Angeleno," 1-MCP treatment before storage at low temperature reduced decay caused by *Monilinia laxa* (Menniti et al., 2004). Also, decay development in apricots was decreased by 1-MCP in a concentration-dependent manner (Dong et al., 2002). In peaches, decay development after inoculation with *P. expansum* was slightly reduced by 1-MCP treatment (Liu et al., 2005). Decay of sweet cherries was also lower in 1-MCP-treated fruit (Mozetic et al., 2006).

7.6 Responses of vegetables

Most of the research on 1-MCP has been concentrated on climacteric fruit. The dearth of research on fresh vegetables may be due to the fact that many vegetables are consumed shortly after harvest rather than being stored for extended periods. Some work has been done on broccoli, carrots, cucumbers, and lettuce. Broccoli is very sensitive to ethylene, which promotes senescence and yellowing. This is delayed by treatment with 1-MCP (Fan and Mattheis, 2000a). On the other hand, yellowing of cucumbers was delayed by 1-MCP only when ethylene was present (Nilsson, 2005). Both Nilsson using cucumbers and Able et al. (2003) examining bok choy and broccoli found that 1-MCP had to be applied immediately after harvest, otherwise its efficacy was greatly reduced. If cucumbers were stored before applying 1-MCP, the treatment was ineffective. Apparently, senescent processes are activated soon after harvest, and once they begin 1-MCP cannot stop their progression.

In carrots and lettuce, treatment with 1-MCP was found to prevent physiological disorders that were associated with ethylene (Fan and Mattheis, 2000b). In carrots, the bitterness due to accumulation of isocoumarin, and in lettuce, the phenols synthesized through the shikimic acid pathway leading to russet spotting were prevented. Potatoes close wounds inflicted during harvesting by developing a layer of suberin, and during this wound healing, ethylene is produced by the tuber. Preventing the production of ethylene by 1-MCP or other inhibitors did not affect suberization (Lulai and Suttle, 2004). Onion bulbs responded to 1-MCP by maintaining higher sugar and dry weight levels (Chope et al., 2007). Sprout growth was reduced by 1-MCP treatment at 4 and 12°C, but not at 20°C. Germination of chayote (*Sechium edule* (Jacq.) Sw), a vegetable native to Middle America, was also prevented by 1-MCP (Cadena-Iniguez et al., 2006).

7.7 Fresh-cut products

The quality of fresh-cut products is influenced by the original quality of the produce at harvest, and maintaining this quality between harvest and preparation, the method of processing, and subsequent handling and storage conditions. A major problem of fresh-cut products is a relatively short postcutting life due to excessive tissue softening and cut-surface browning. These processes are stimulated by ethylene. Vegetables are more often prepared and sold as fresh cut, minimally processed or ready-to-eat, and lettuce is one of the major products prepared in this way. The browning in shredded lettuce is due to an increased synthesis of phenolic compounds. The application of 1-MCP to lettuce before minimal processing resulted in a reduction of russet spotting of ribs as well as cut-edge browning (Saltveit, 2004; Tay and Perera, 2004). However, if the application was made after cutting, the increase in phenolic compounds was not affected. Other vegetables and herbs may also benefit from 1-MCP treatment. Although most vegetables are nonclimacteric and are generally not stored for extended periods, it has been shown that even low levels of ethylene can significantly decrease their shelf life (Ku et al., 1999a). Therefore, 1-MCP may be beneficial even on these commodities.

The wound response of fresh-cut products induces a transient elevation of ethylene in the tissue. Cucumbers treated with 1-MCP before slicing had a greater retention of firmness and better surface color, even when exposed to ethylene after slicing (Lima et al., 2005). The response was cultivar dependent, with firmer cultivars benefiting less than those that were less firm. This has led Nilsson (2005) to suggest that cucumber may not benefit from 1-MCP unless ethylene is present.

Harvested leafy vegetables and herbs also benefit from 1-MCP with slowing of senescence-associated leaf yellowing. Mint (Kenigsbuch et al., 2007), rocket (Kourkounaras et al., 2006), parsley (Lomaniec et al., 2003), and coriander (Jiang et al., 2002) all had longer shelf life after treatment with 1-MCP, even when stored in the presence of ethylene. Both chlorophyll and protein degradation leading to amino acid accumulation were lower in treated leaves. Interestingly, in detached coriander, mint, and parsley leaves both ethylene and respiration were higher with 1-MCP than in control leaves, although senescence was retarded (Jiang et al., 2002; Lomaniec et al., 2003; Kenigsbuch et al., 2007).

Asian vegetables such as Chinese mustard, choy sum, garland chrysanthemum, mibuna, mizuna, and tatsoi are often sold in minimally processed packages. The leaf yellowing due to senescent processes is prevented with pretreatment with 1-MCP (Able et al., 2003). The greatest effect was when ethylene was present during the shelf life period. Without ethylene 1-MCP had a minimal effect on all the vegetables except mizuna and mibuna where natural yellowing was delayed. Pak choy also had longer shelf life after 1-MCP treatment only when ethylene was present (Able et al., 2002).

Tomato is climacteric and increases in ethylene occur as a result of ripening as well as wounding. Light-red tomatoes responded to 1-MCP treatment, and slices prepared from these fruits maintained firmness better and did not develop waterlogging when held at 5°C, while slices from control tomatoes lost firmness (Jeong et al., 2004). Treating red tomatoes with 1-MCP before slicing gave no benefit or extension of shelf life.

The application of 1-MCP in fresh-cut apples decreased the ethylene production, respiration, softening, color change, and synthesis of aroma compounds (Jiang and Joyce, 2002; Bai et al., 2004; Calderon-Lopez et al., 2005). In pineapple, 1-MCP decreased respiration,

browning, loss of visual quality, lightness, and ascorbic acid (Budu and Joyce, 2003). Ergun et al. (2006b) reported that slices made from 1-MCP-treated papayas had double the shelf life than slices made from untreated papayas. "Galia" melon cubes had reduced water soaking and better firmness when treated with 1-MCP before slicing (Ergun et al., 2007). Fresh-cut watermelon slices stored longer under modified atmosphere at 5°C when fruits were treated with 1-MCP before slicing (Saftner et al., 2007). Fresh-cut banana has a short shelf life due to fast browning and softening after processing. 1-MCP treatment of slices decreased the rate of softening and respiration rate, but browning rates were not affected (Vilas-Boas and Kader, 2006). To control browning, a dip in antioxidants was required. In other fruits such as kiwifruit, persimmon and mango, Vilas-Boas and Kader (2007) found different responses in firmness, color, and respiration and ethylene production depending on the timing of the 1-MCP application. In general, giving the 1-MCP after slicing had a stronger effect than giving it to the whole fruit, and giving the 1-MCP together with a $CaCl_2$ dip had a synergistic effect on slice firmness. A similar study with strawberries also found a synergistic effect with 1-MCP and a $CaCl_2$ dip (Aguayo et al., 2006). In strawberry, 1-MCP by itself, either to whole strawberries or to slices had no beneficial effect on firmness or appearance. Strawberry is the only nonclimacteric fruit that has been reported on so far for fresh cut.

7.8 Conclusions

The discovery and subsequent commercialization of 1-MCP has provided exciting opportunities for postharvest scientists to gain insight into the fundamental processes that are involved in ripening and senescence of fruit and vegetables. For products such as vegetables and nonclimacteric fruit where further senescence, such as yellowing, will decrease quality, 1-MCP applications that prevent change are desirable. For climacteric fruit, success on a commercial scale will be based on delaying rather than preventing ripening, in order to extend shelf life but eventually achieve a full ripe product. In the area of basic research, the availability of 1-MCP is likely to have a dramatic impact on our understanding of the involvement of ethylene in plant metabolism and in plant–pathogen interactions. It is an exciting tool to use, and much knowledge can be gained from its application.

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Chapter 8

Structural Deterioration of the Produce: The Breakdown of Cell Wall Components

Pradeep S. Negi and Avtar K. Handa

8.1 Introduction

Fruit ripening is a dynamic transitional period during which many easily perceived changes, such as alterations in pigmentation, firmness, sweetness, and acidity take place. These changes make fruit desirable for human consumption and capable of seed dispersal by birds, animals, and environments. Fruit firmness is associated with several attributes including crispness, mealiness, grittiness, chewiness, succulence and juiciness, fibrousness, toughness, and oiliness. Additionally, development of various organoleptic components such as sweetness, sourness, astringency, bitterness, and production of volatile compounds leading to characteristic aroma is connected with fruit textural changes. Although most of these changes impart desirable traits to various fruits and vegetables, some of the fruit softening associated changes make them unacceptable for marketing. These include development of off-flavors and off-odors with excessive softening of tissues. Textural softening can also increase susceptibility to phytopathogens due to their proneness to solute leakage that provide rich media for their growth, and resulting in severe losses during postharvest storage and marketing (Prasanna et al., 2007).

Large economic losses results from inability to retard ripening-associated excessive softening of fruits between harvest and marketing. These losses occur due to culling of perishable commodities at field and packinghouses, grading, storage, transit, retail, and consumer. In developing countries, theses losses can range between 10 and 100%, especially due to phytopathogen-related tissue rotting of certain commodities. The economic consequences of postharvest fruit softening have led to considerable interests of geneticists, physiologists, biochemists, and in recent year's molecular biologists to understand the molecular basis of fruit softening. The last 40 years have seen a significant increase in our understanding of biochemical changes associated to fruit textural modifications. Emerging recombinant DNA technologies including reverse genetics have begun to provide some answers. In this chapter we have described the relationship of cell wall chemistry and various families of cell wall–modifying enzymes to the developmentally regulated softening of fruit during ripening. Also discussed are various postharvest factors affecting structural deterioration of fruit crops and potential of chemical or genetic means to reduce crop losses.

8.2 Fruit softening

Ripening-associated fruit softening is usually represented by a decrease in the firmness of the tissues involving modifications to the polysaccharide components of the primary cell wall and middle lamella that cause a weakening of the structure. This interplay between primary cell wall and middle lamella components is a very complex process involving many families of cell wall-modifying enzymes. Additionally, structural proteins such as expansin also play role (Brummell, 2006; Vicente et al., 2007). Developmental regulation of expression of many of the genes encoding the cell wall-modifying enzymes underlying this process has been demonstrated. Many of these proteins are expressed at the onset of fruit ripening and secreted into the extracellular spaces from the symplast. In addition to developmentally regulated fruit softening, other mechanisms such as water relations involving turgor pressure and free radicals may also contribute to fruit softening (Fry et al., 2001; Dumville and fry, 2003).

Softness and textural characteristics of ripe fruit have been suggested to be determined by the ratio between the declining firmness of primary cell wall and the declining strength of the intercellular adhesion (Harker et al., 1997). It has been suggested that both the cell wall and the middle lamella must weaken for fruit to change from hard unripe to soft/crisp and yet juicy (Brummell, 2006). Relatively robust intracellular connections with the weakening of the primary cell walls would keep fruit firm and crisp. On biting, cells in such fruit will split open resulting in release of cellular contents and making fruit juicy when chewed. Cell separation due to breaking of the intracellular connection would result in fruit that is both soft and juicy. In case the primary cell walls remain strong and the intracellular adhesion is too weak, the fruit tissue will be soft with an unpleasant dry texture as observed in apple and peach injured by chilling (Harker and Hallett, 1992; Brummell et al., 2004b; Brummell, 2006). Overripe fruit exhibit loss of both primary cell walls and intracellular connections. However, detailed characterization of the structural and compositional changes is needed to strengthen this hypothesis.

8.3 Structure and composition of primary cell walls in fruits

The primary wall is important for structural and mechanical support of the plant body. It maintains and determines cell shape and form, resists internal turgor pressure of cell, controls rate and direction of growth, regulates diffusion of material through the apoplast, carbohydrate storage, and provides protection against pathogens, dehydration, and other environmental factors. Besides polysaccharides, a range of structural and enzymatic proteins, hydrophobic compounds, and inorganic molecules also exist in cell wall. The primary cell wall is highly hydrated, and the aqueous components contain various dissolved solids, ions, and soluble proteins including enzymes. Several models for plant cell walls have been proposed. These models are based on the same common fundamental components (Table 8.1) but differ in terms of how these components interact with each other. Interactions proposed among various components include cellulose microfibrils cross-linked with hemicellulose with pectin acting as cement, Ca^{2+} bridges connecting uronic acid carboxyl function and borate diesters of two rhamnogalacturonan II monomers, covalent cross-linkage among different classes of pectin to form a single heterogeneous network, covalent bonding between pectin and xyloglucan, as well as pectin and

Table 8.1 Common polymers of primary cell wall and their structures

Polymer	Molecular structure
Cellulose	(1→4)β-D-glucan chains held together with hydrogen bonding, forming very long crystalline microfibrils
	Cross section contains 36 glucan chains
Xyloglucan	Backbone similar to cellulose, i.e., (1→4)β-D-glucan
	Regular substitution on three out of four consecutive glucose residue with α-D-xylose
	Xylose occasionally extended with β-D-galactosyl-α-L-fucose or α-L-arabinose in some species
	The reducing end of unsubstituted glucose residues is susceptible to cleavage by *Trichoderma* endo-(1→4)β-D-glucanases (EGases) producing similar amounts of heptasaccharide (Glc4.Xyl3) and nonasaccharide (Glc4.Xyl3.Gal.Fuc) xyloglucan subunit oligosaccharides
Glucomannan	Backbone contains regions of (1→4)β-D-glucan and (1→4)β-D-mannan in nearly similar amounts galactomannan
	Occasionally terminal has a side chains of single unit of α-D-galactose
Glucuronoarabinoxylan	Backbone of (1→4)β-D-xylan
	Side chains of single unit of nonreducing terminal α-L-arabinose and α-D-glucuronic acid
Homogalacturonan	Made of long chains of (1→4)α-D-galacturonic acid
	Initially highly methyl-esterified
Rhamnogalacturonan I (RG-I)	Made of alternating α-D-rhamnose and α-d-galacturonic acid residues; long side chains of either unbranched (1→4)β-D-galactan or branched α-L-arabinans or type I arabinogalactans attached to the rhamnose residues
Rhamnogalacturonan II (RG-II)	Backbone made of (1→4)α-D-galacturonic acidlike homogalacturonan; complex side chains of different types of neutral sugar. A minor cell wall component. RG-II monomers can dimerize together as boron diesters and may affect the cell wall porosity
Structural proteins	Four different types including expansin; some are heavily glycosylated

cellulose. These models have provided useful starting points to understand ripening-associated changes in cell wall, that lead to change in fruit texture (Vincken et al., 2003; Brummell, 2006; Vicente et al., 2007).

Cellulose microfibrils are composed of unbranched (1,4)-linked α-D-glucan and synthesized in the plasma membrane by large hexameric complexes. Long crystalline ribbons of about 3–5 nm width of cellulose microfiber, formed by extensive hydrogen bonding among several parallel glucans chains, provide cell wall mechanical strength and resistance to enzymatic attack. The matrix glycans (hemicelluloses) are neutral or weakly acidic, composed mainly of neutral sugars, and do not contain galacturonic acid (GalA). The basic structure of hemicellulose is similar to cellulose, but does not form microfibrils due to its branching and other sugar modifications. Hemicelluloses are synthesized in the Golgi apparatus and deposited to the wall surface by transport through vesicles. Xyloglucan and arabinoxylan predominate among the various components of hemicellulose. The xyloglucan backbone is similar to that of cellulose but has numerous regularly spaced xylose side chains, some extended with either Gal-Fuc or Ara. The arabinoxylan backbone is

made of a (1,4)-α-D-xylan with occasional single glucuronic acid and arabinose side chain. Other sugars attached to arabinoxylans include glucuronic acid and ferulic acid esters. Glucomannan (alternating regions of 1,4-β-D-glucan and 1,4-β-D-mannan), one of the other components of the primary cell wall, may have roles similar to xyloglucan and arabinoxylan.

Hemicelluloses present in the ripening fruits include glucuronoarabinoxylans, xylans, and glucomannans that are loosely associated with cellulose microfibrils that are long, rigid, inextensible fibers.

Pectins represent a complex and heterogeneous group and can contain as many as 17 different monosaccharides (Vincken et al., 2003). Like hemicellulosic components, pectins are also synthesized in the Golgi apparatus and deposited to the wall surface via vesicles. Pectin polymers are broadly divided into several distinctive categories that include galacturonan, rhamnogalacturonan I (RG-I), and arabinogalactan II (AG-II), with each category having further subgroups. Galacturonan shares a backbone of 1,4-linked α-D-Gal$p$A (galacturonic acid polymer) residues and includes homogalacturonan (HG), rhamnogalacturonan II (RG-II), and xylogalacturonan (XGA). Homogalacturonans are initially synthesized and secreted into plant cell wall with a high degree of methylesterification (Carpita and McCann, 2000), which declines during development due to the action of apoplastic pectin methylesterase (Willats et al., 2001a). Some of the GalA residues of HG can be methylesterified at C-6 or carry acetyl moiety on O-2 and O-3 position. RG-II contains a few rhamnose residues that are present only in the side chains and not in the backbone. The branched galacturonan XGA contains β-D-Xylp-(1$\rightarrow$3) side chains with the degree of xylosylation varying between 25 and 75% in watermelon and apple, respectively (Vincken et al., 2003). Some of the GalA residues of XGA can be methylesterified. The methylesterification of HG plays significant role in processing attributes of fruits, in particular (Thakur et al., 1996a, b), and the industrial properties of pectins, in general (Thakur et al., 1997).

The RG-I backbone contains repeating disaccharide unit [$\rightarrow$2)-α-L-Rha$p$-(1$\rightarrow$4)-α-D-Gal$p$A-(1$\rightarrow$]$_n$ where the n can be larger than 100. Like RG-II, some of the RG-I galacturonyl residues can be acetylated at O-2 and O-3 and the rhamnosyl residues substituted with neutral sugars at O-4. Although RG-I can exist as unbranched molecule, generally 20–80% of Rha are branched. RG-I may contain single (α-D-Gal$p$-(1$\rightarrow$4)) as well as polymeric side chains such as arabinogalactan I (AG-I) and arabinan (50 glycosyls or more residues). The AG-I backbone is composed of a 1,4-linked α-D-Gal$p$ and α-L-Ara$f$ residues. The backbone of arabinans is consist of a 1,5-linked α-L-Ara$f$ with possible substitutions with α-L-Ara$f$-(1$\rightarrow$2)-, α-L-Ara$f$-(1$\rightarrow$3)-, and/or α-L-Ara$f$-(1$\rightarrow$3)-α-L-Ara$f$-(1$\rightarrow$3) side chains. The hairy pectins that vary with plant species include mostly complexes of RG-I, AG-I, and arabinan. It is not yet established if the arabinogalactan II (AG-II) is part of the pectic complex. AG-II is primarily associated with arabinogalactan proteins (AGPs) and often coextracted with pectin suggesting covalent link between these moieties. The backbone of AG-II contains 1,3-linked β-D-Gal$p$ with short side chains of α-L-Ara$f$-(1$\rightarrow$6)-[α-D-Gal$p$-(1$\rightarrow$6)]$_n$ with $n = 1$, 2, or 3. The galactosyl residues of the side chains can be substituted with α-L-Ara$f$-(1$\rightarrow$3) residues. Amount of structural proteins is very low, and it ranges from 1 to 10% on dry weight basis. AGPs contain over 90% polysaccharides and the protein moiety rich in Pro/Hyp, Ala, Ser, and Thr (Vincken et al., 2003).

8.4 Ripening-associated cell wall changes

Fruit ripening is associated with extensive structural alterations within its semisoluble pectin matrix, the pectin-rich middle lamellae that cement walls of adjacent cells, and the hemicellulose–cellulose network. Pectins, hemicelluloses, cellulose, and starch are the common polysaccharides that undergo modifications during ripening. Degradation of pectins that represent over 50% of total carbohydrate in the fruit cell walls has been implicated in tissue softening of a number of fruits. Pectin chemistry plays important roles in cell wall hydration status and the mobility of resident enzymes by changing cellular porosity and ion-facilitated gel formation. Even before the onset of fruit ripening, a loss of pectic galactan side chains and the depolymerization of matrix glycans are usually initiated, which is followed by a loss of pectic arabinan side chains. Fruit ripening initiates the depolymerization of pectins, which becomes very prominent during the late ripening. The relationship between the pectic material degradation and the decrease in the firmness of fruit tissues has been extensively documented. In addition to the pectin degradation, xyloglucan breakdown has been reported in the early stage of softening, but it is relatively limited and more consistent. The degradation of both xyloglucans and polyuronides is cooperatively involved in fruit softening processes; the xyloglucan breakdown may contribute to the initiation process, while the polyuronide degradation to the excessive softening process (Wakabayashi, 2000). Slippage between hydrogen-bonded hemicellulose and cellulose polymers also contributes to softening (Brummell et al., 1999; Powell et al., 2003). In part, the expansin family of cell wall proteins (Cosgrove, 2005) mediates such cell wall "creep," and it is postulated that the hydrogen-bonded interface between cellulose and xyloglucan may act as a substrate for these bindings (Rose and Bennett, 1999; Brummell and Harpster, 2001).

Significant differences in ripening-associated cell wall polysaccharide modifications exist among species of the same genus and even among different cultivars of same species (Hiwasa et al., 2004; Rosli et al., 2004). These differences along with the marked diversity in cell wall changes between species greatly contribute to the vast variability in firmness, texture, and juiciness characteristics observed among different ripe fruits. Emerging evidences have begun to support hypothesis that different cell wall changes are a result of variations in the abundance, activity, timing, and type of gene family members of various polysaccharide-modifying enzymes expressed during ripening. This is likely responsible for the different cell wall processes observed between soft and crisp fruits. In spite of the similar proportions of cell wall material, the melting and structure of the cell walls in these fruits are very different at the ripe stage. Solubilization of the middle lamella and a restructuration of the primary cell walls arising from the cells separation were observed in crisp fruits, whereas the middle lamella of the soft fruits is better preserved and the primary cell walls are thin. The changes in texture of cherries during ripening are linked to cell wall degradation, involving synthesis and degradation of polymers, and the fruit texture depends on the extent of the links between cell wall polymers. An increase in pectin solubility leads to cell sliding and an elastic aspect of tissues (Batisse et al., 1996). Figure 8.1 shows changes in relative solubilization of various major cell wall polysaccharides during ripening of some of the fruits.

8.5 Pectin solubilization

During fruit softening, pectin typically undergoes solubilization followed by depolymerization that is thought to contribute to wall loosening and disintegration resulting in textural

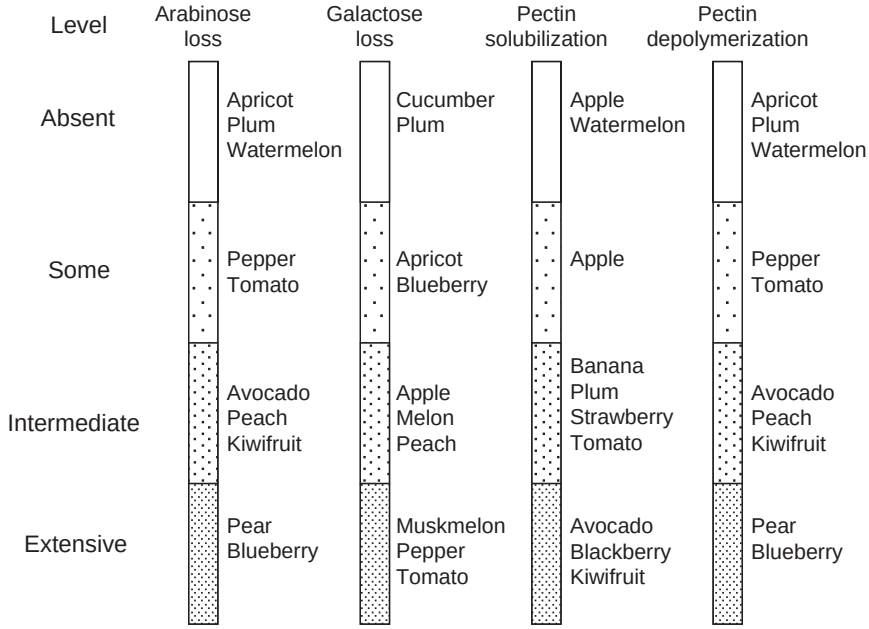

Fig. 8.1 Classification of fruits based on extent of cell wall polysaccharide modifications during ripening. Shown are ripening-associated losses of arabinose and galactose and solubilization and depolymerization of pectic component from cell walls of fruits of various species. Levels represent: Extensive >70%, intermediate 25–70%, some ≤25%, and absent undetectable. (Redrawn from Brummell, 2006.)

changes. Substantial differences in proportion of pectin solubilization exist between species; greatest solubilization occurring in ripening avocado followed by kiwifruit and blackberry, whereas solubilization was very low or absent in apples and watermelon (Brummell, 2006). The increased pectin solubilization is correlated with a swelling of cell wall (Redgwell et al., 1997a). In persimmon, avocado, blackberry, strawberry, and plum fruit that ripens to a soft melting texture, wall swelling was pronounced, particularly in vitro. In vivo swelling was marked only in avocado and blackberry. Fruit that ripened to a crisp, fracturable texture such as apple, pear, and watermelon did not show either in vivo or in vitro swelling of the cell wall. There is a correlation between swelling and the degree of pectin solubilization, suggesting that wall swelling occurred as a result of changes to the viscoelastic properties of the cell wall during pectin solubilization. The pectin that is solubilized during ripening generally has a low Gal (Redgwell et al., 1997b) and Ara content (Brummell et al., 2004a). This indicates that either solubilization and Gal/Ara loss largely affects different pectic molecules (Redgwell et al., 1997b) or loss of almost complete side chain results in polyuronide solubilization. Swelling was associated with movement of water into voids left in the cellulose–hemicellulose network by the solubilized pectin (Redgwell et al., 1997a). These processes combined with the loss of pectic side chains increase wall porosity that may allow increased access of degradative enzymes to their substrates later in ripening (Brummell, 2006). The walls of fleshy fruits become much more open, and hydrophilic environment exists as ripening progresses. The increased accessibility of hydrolytic enzymes to their substrate further promotes polymer dismantling.

Polyuronides in tomato and avocado exhibit progressive depolymerization from unripe to midripe to full ripe, whereas in peach there was a little change in midripe fruit compared with unripe but it showed a dramatic depolymerization at the full ripe stage. Depolymerization of polyuronides has important consequences for the strength of cell-to-cell connections, which affects softening and texture of the fruit, but the large differences in the extent of polyuronide depolymerization among species during ripening indicates that altered intercellular adhesion is more important component of textural changes in some species than others. Induction of polyuronide depolymerization in a nonsoftening mutant tomato by expressing polygalacturonase (PG) has shown that it alone is not the determinant of fruit softening (Giovannoni et al., 1989). Depolymerization of polyuronides late in ripening is a major contributor to weakening of the middle lamella and declining intercellular adhesion. Softening often does not correlate with polyuronide depolymerization early in ripening, although the more extensive depolymerization occurring late in ripening is associated with a large decline in firmness.

A reduction in degree of pectin methylesterification is a common feature of most aspects of plant development, which is most noticeable during fruit ripening. A large decrease in the degree of pectin methylesterification has been reported during ripening of kiwifruit (Redgwell et al., 1990), papaya (Paull et al., 1999), avocado (Wakabayashi et al., 2000), peach (Brummell et al., 2004a), and grapes (Barnavon et al., 2001). A 40% drop in the degree of pectin esterification was reported in fruit development in tomato cv VF145b-7879 (Koch and Nevins, 1989), but was not observed in other tomato varieties (Tieman et al., 1992). Demethylesterification of pectin not only affects the rigidity of the pectin network and properties of the wall but also modifies the diffusion and activity of enzymes within the wall spaces. The changing ionic conditions in the apoplast (alteration in pH, increase in K ion, and osmoticum) may affect the activity of the cell wall hydrolases (Grignon and Sentenac, 1991; Chun and Huber, 1998; Almeida and Huber, 1999) and aggregation of pectin molecule (Fishman et al., 1989).

During ripening of avocado, water-soluble polyuronides increased dramatically, concomitant with marked downshifts in molecular mass. PG plays the central role in polyuronide degradation in ripening avocado fruit cell walls, and partial deesterification is necessary for the increase in the susceptibility of polyuronides to PG (Wakabayashi et al., 2000).

Fleshy fruit of strawberry soften during ripening mainly as a consequence of solubilization and depolymerization of cell wall components and is associated with an increment of pectin solubility and a reduction of the molecular mass of hemicelluloses (Martinez et al., 2004). The amount of water-soluble polymers (WSP) increased from small green (SG) to white (W) stage, on the contrary, the hydrochloric acid–soluble pectins (HSPs) decreased during ripening. The amount of hemicellulosic polysaccharides and cellulose also decreased. A slight depolymerization was observed in hemicelluloses (Rosli et al., 2004).

8.6 Matrix glycan solubilization

Galactan and arabinan play important role in cell wall structure and function. The majority of Gal and Ara in the primary walls is present as the 1,4-β-D-galactan, 1,5-α-L-arabinan, and branched arabinogalactans side chains of RG-I with smaller amounts in structural

glycoproteins and in the side chains of xyloglucan, glucuronoarabinoxylan, and RG-II (Carpita and Gibeaut, 1993). Debranching of RG-I side chains appear to be an important component of changes that alter fruit firmness. During ripening, loss of polymeric Gal and Ara from the cell wall takes place, the extent of which is dependent on species (Gross and Sams, 1984; Redgwell et al., 1997b). The appearance or disappearance of the 1,4-β-D-galactan was correlated with wall firmness in pea cotyledons (McCartney et al., 2000) and potato (Ulvskov et al., 2005).

Cell wall arabinan is necessary for maintaining cell wall flexibility, as its degradation prevented the cell wall movements necessary for stomatal opening and closing (Jones et al., 2003). Arabinan is also essential for normal intercellular adhesion, as a mutant in Nicotiana lacking firmly bound arabinan had loosely attached cells (Iwai et al., 2001). Reduced deposition of arabinan in the regions surrounding intercellular spaces was correlated with weakened cell-to-cell contacts in *Cnr* mutant of tomato, which does not ripen properly (Orfila et al., 2001). Retention of cell wall Ara correlated with the altered intercellular adhesion and increased wall strength in mealy peaches (Brummell et al., 2004b).

Depolymerization of matrix glycans from cell wall is closely correlated with fruit softening. During ripening a progressive downshifts in the molecular weight of matrix glycans have been observed in tomato (Tong and Gross, 1988; Maclachlan and Brady, 1994; Brummell et al., 1999), pepper (Gross et al., 1986), melon (Rose et al., 1998), strawberry (Huber, 1984), avocado (Sakurai and Nevins, 1997), kiwifruit (Redgwell et al., 1991), and in many other species. The depolymerization occurs in both the xyloglucan component and total matrix glycans. Depolymerization of matrix glycan is thought to be a major contributor to the reduced rigidity of cell wall and fruit softening. Due to its important structural role of cross-linking cellulose in wall, depolymerization of xyloglucan may be one of the important factors along with other polymer degradation. Loosening of the xyloglucan–cellulose network may be also a part of cell wall swelling, which is correlated with pectin solubilization.

In general, depolymerization of matrix glycans begins during early ripening and continues throughout, and these changes correlate with softening. Depolymerization may be very slight (apple, strawberry, banana, and bell pepper); progressive, which begins slowly during ripening but exhibits substantial depolymerization late in ripening (kiwifruit, tomato avocado, and papaya); or abrupt, absent in early ripening and occurs rapidly during late ripening (melon and melting flesh peach). The progressive loss of large arabinan and galactan side chains of RG-I during ripening is likely to alter cell wall rigidity/flexibility and intercellular attachment, increase cell wall porosity, and may affect pectin solubilization.

8.7 Enzymatic regulation of polyuronide depolymerization

During ripening, multiple enzymes promote disassembly of individual cell wall polysaccharides. Most of the disassembly of the cell wall during ripening is due to the actions of a range of polysaccharide-modifying enzymes secreted into the cell wall space from the symplast, although nonenzymatic mechanisms mediated by free radicals may also be involved (Fry et al., 2001, 2002; Dumville and Fry, 2003). The expression of many genes increases during ripening, and the product of these genes may influence depolymerization or

modifications of cell wall polysaccharides (Alba et al., 2005; Handa et al., 2007). For example, the expression of LePG in ripening tomato is accompanied by increases in other proteins whose functions are to depolymerize or modify polysaccharides in cell wall, such as pectin methylesterase (PME) (Harriman et al., 1991), endo-1,4,β-glucanase (Lashbrook et al., 1994), xyloglucan endo transglycosylases (Arrowsmith and de Silva, 1995), β-galactosidases (Smith et al., 1998; Carey et al., 2001), and pectate lyases (Marin-Rodriguez et al., 2002).

Pectin modification provides an excellent example of the modification of polysaccharide domains. A number of enzymes modify or degrade pectin including enzymes that act on pectins such as exo- and endo-acting PGs, PMEs, β-galactosidases, and pectate lyases (PLs) (Table 8.2). Most of these enzymes exist in multigene families with a subset of one or more gene family members regulating the cell wall modification processes associated with fruit ripening. Complex interactions between gene family members of each enzyme class and between different enzymes may also occur. A well-characterized cooperative relationship exists between PG and PME. PME plays critical roles in establishing structurally and functionally distinct classes of pectin, important in many stages of plant growth and development (Ridley et al., 2001; Willats et al., 2001a).

Pectin is the most abundant class of macromolecules within the cell wall matrix and in the middle lamella between primary cell walls. During fruit softening, pectin typically undergoes solubilization and depolymerization processes that are thought to contribute to wall loosening and disintegration. PG, which catalyzes the hydrolytic cleavage of galacturonan linkage of pectin, is the most abundant pectin-degrading enzyme whose action coincides

Table 8.2 Pectin-modifying and degrading enzymes in fruits

Substrate	Enzymes	Products
Pectin	Pectin methyl esterase	Pectic acid + methanol
	Endopolymethyl galacturonase	Methyl oligogalacturonides
	Rhamnogalacturonase	α-(1,2)Linked L-Rha, α-(1,4) linked D-Gal
	Endopectin lyases	Unsaturated oligogalacturonides
Hairy pectin	Rhamnogalacturonan acetylesterase	Pectin + acetic acid
	Pectin acetyl esterase	Pectin + acetic acid
Smooth pectins	Lyases	Oligogalacturonides
Protopectin	Protopectinase	Pectin
Pectic acid	Endo-PG	Oligogalacturonides
	Exo-PG	Monogalacturonides
	Endopectate lyases	Oligogalacturonides
	Exopectate lyases	Unsaturated digalacturonides
Trigalacturonic acid	Oligogalacturonide hydrolase	Monogalacturonides
Δ4:5 (galacturonide)n	Δ4:5 unsaturated oligogalacturonide hydrolase	Unsaturated monogalacturonide + galacturonides ($n-1$)
Unsaturated digalacturonate	Oligogalacturonide lyases	Unsaturated monogalacturonides
Arabinans	α-L-Arabinofuranosidase	α-L-Arabinose
(1,5)-α-Arabinans	Endoarabinanase	Arabinose and higher oligosaccharides
Galactans	β-D-Galactanase	β-D-Galactose

with fruit softening (DellaPenna et al., 1986; Biggs and Handa, 1989; Brummell and Harpster, 2001). However, molecular genetic studies have shown that even though the PG is responsible for polyuronide depolymerization and solubilization, it makes only a partial contribution to fruit softening.

Structural alterations in the cell walls of ripening fruit take place within its semisoluble pectin matrix and within the pectin-rich middle lamellae that cement walls of adjacent cells. Pectins in fruits represent over half of the cell wall polysaccharides present (Brummell and Harpster, 2001). Ripening-associated changes in pectin porosity and ion-facilitated gel formation regulate cell wall hydration status and the mobility of resident enzymes. Biologically active cell wall fragments are generated during plant processes, including defense against pathogens (Vorwerk et al., 2004).

Homogalacturonans are initially synthesized and secreted into plant cell wall with a high degree of methylesterification (Carpita and McCann, 2000), which declines during development due to the action of apoplastic pectin methylesterase (PME) (Willats et al., 2001a). A reduction in degree of pectin methylesterification is a common feature of most aspects of plant development, which is most noticeable during fruit ripening. Large decrease in the degree of pectin methylesterification has been reported during fruit development in tomato (Koch and Nevins, 1989; Tieman et al., 1992), kiwifruit (Redgwell et al., 1990), papaya (Paull et al., 1999), avocado (Wakabayashi et al., 2000), peach (Brummell et al., 2004a), and grapes (Barnavon et al., 2001). In general, alkaline PMEs from plants are thought to deesterify contiguous Gal residues in homogalacturonans by the so-called single chain mechanism (Denes et al., 2000), in which a PME deesterifies contiguous Gal residues in a pectin chain in a linear fashion to produce blocks of pectin with free carboxyl groups. PMEs can deesterify more limited runs of Gals using a multiple attack mechanism also (Grasdalen et al., 1996; Denes et al., 2000). PME action pattern affects block length of deesterified HGA and its propensity for calcium-cross-linked gel formation.

PMEs may play important roles in determining the extent to which demethylated polygalacturonans are accessible to degradation by PGs, releasing galacturonic acid (exo-PG) or oligogalacturonate (endo-PG), and the availability of homogalacturonan carboxylic group for calcium ion binding, resulting in supramolecular assemblies and gels. The formation of these calcium-mediated pectin gels significantly affects the mechanical properties of cell wall and adds rigidity to the wall (Thakur et al., 1997; Brummell and Harpster, 2001; Rose et al., 2003). Although PMEs play a little role in fruit softening during ripening, their effect on tissue integrity is substantial (Brummell and Harpster, 2001). Low PME expression in Cnr tomato mutants is speculated to be responsible for maintaining a strong cell wall, highlighting its role in maintaining fruit cell wall integrity (Eriksson et al., 2004).

Peach genotype that show limited flesh softening during ripening were characterized by lower losses of neutral sugars, especially those of arabinose and galactose, higher ethylene production during ripening, higher levels of uronic acids, and increased capacity for calcium binding in the water-insoluble pectin fraction compared with fruits of the extensive flesh-softening genotypes during ripening. The limited softening character can be attributed to the decreased activity of PME combined with higher levels of calcium in the water-insoluble pectin fraction and reduced solubility of cell wall pectin (Manganaris et al., 2006). During ripening of nectarine, there was a temporal coincidence among higher rates of ethylene production, higher PME and PG activities, and lower firmness. PME and PG activities

increased during ripening, with a highly negative linear correlation between activities of the two enzymes and firmness (Artes and Salmeron, 1996).

In rambutan the firmness of skin increased toward harvest with decrease in water-soluble pectic substances, hemicellulose, and cellulose. Although PE activity in the pulp did not change during fruit development, endo-PG increased toward harvest, suggesting that endo-PG may relate to cell wall polyuronide degradation in the pulp (Kondo et al., 2002).

An overall increase of cell wall material was observed during green bean pod development, and major changes were detected in the pectic polymers. Initially, cell walls contained large amounts of neutral, sugar-rich pectic polymers (rhamnogalacturonan), and during elongation, more galactose-rich pectic polymers were deposited into the cell wall, and the level of linear homogalacturonan steadily increased. During maturation of the pods, galactose-rich pectic polymers were degraded and there was an increase in the amount of ionically complexed pectins at senescence. The most abundant enzymes were PME, peroxidase, β-galactosidase, and α-arabinosidase with PG present only in very small amounts throughout pod development (Stolle-Smits et al., 1999). Ebbelaar et al. (1996) also observed low endo-PG or exo-PG activities in early developmental stages, while PE activities were measurable during all stages of pod and seed development. These results do not favor a possible synergistic action of PE and PG. PE gene expression levels varied significantly in pods from different cultivars suggesting its involvement in determining pod morphology.

Pectate lyases are the other pectin-degrading enzymes that randomly cleave β (1–4) linkages between galacturonosyl residues, generating 4,5-unsaturated oligogalacturonates by β-elimination (Willats et al., 2001b). In strawberries an antisense PL gene caused an increase in fruit firmness and reduced the postharvest softening, without affecting weight, color, and soluble solid content of the fruit, indicating that PLs play an important role in fruit softening (Jiménez-Bermúdez et al., 2002). In ripening strawberry, accumulation of a PL gene product is absent in green fruit but subsequently appears at the white stage and further during ripening (Medina-Escobar et al., 1997). Constitutive downregulation of corresponding gene expression in transgenic strawberry reduced softening as measured by the presence of higher numbers of red fruit within fruit populations showing enhanced internal and external firmness (Jiménez-Bermúdez et al., 2002). Firmness of transgenic fruit was preferentially enhanced during the developmental transition from white to red fruit, with red transgenic fruit populations being approximately twofold firmer than populations of ripe nontransformed controls. Populations of ripe transgenic fruit also maintained greater levels of overall firmness during several days of postharvest storage (Jiménez-Bermúdez et al., 2002).

The action of the endogenous enzyme from banana pulp tissue revealed a significant increase in calcium-dependent pectate lyase activity during ripening. The enhanced levels of enzyme activity corresponded with an increase in soluble polyuronides from banana pulp (Marin-Rodriguez et al., 2003).

In some fruit, pectin solubilization can also be nonenzymatic. In ripening kiwifruit, it is proposed that pectin solubilization may be due to nonenzymically regulated cell wall swelling (Newman and Redgwell, 2002). It has also been proposed that ascorbate, copper ion, and H_2O_2, naturally produced in cell walls, can solubilize fruit pectin via the production of hydroxyl radicals (Dumville and Fry, 2003).

8.8 Enzymatic regulation of galactose loss during ripening

During ripening, fruits exhibit a large decrease in galactose from their cell wall polymers (Gross and Sams, 1984). In tomato, the decline in galactose starts early and increases with ripening, and occurs primarily from pectic fraction with matrix glycan and cellulose fractions showing only a slight loss (Gross, 1984; Seymour et al., 1990). Most of the galactose is a part of the side chains attached to rhamnose of the rhamnogalacturonan (RG-I) backbone and is linked either in type I $(1\rightarrow4)\beta$-D-galactan chains or in type II branched $(1\rightarrow3)$,$(1\rightarrow6)\beta$-D-galactan chains (Carpita and Gibeaut, 1993). It is the $(1\rightarrow4)\beta$-D-galactan that is depolymerized during ripening of tomato fruit (Seymour et al., 1990). Due to the lack of endogalactanases in higher plants, the exo-β-D-galactosidase (EC 3.2.1.23) enzyme activity has been implicated in depolymerization of β-galactan present in cell wall. Exo-β-D-galactosidase catalyzes hydrolysis of the terminal nonreducing β-D-galactosyl residues from β-D-galactosides. Multiple forms of β-D-galactosidase are present in fruits. Among the three forms designated as β-galactosidase I, II, and III, only the β-galactosidase II is active against a $(1\rightarrow4)\beta$-D-galactan-rich polymer prepared from tomato cell walls (Pressey, 1983). It also exhibits activity against a variety of galactoside substrates indicating a β-D-galactosidase/exogalactanase activity (Smith and Gross, 2000). Whereas β-galactosidase I and III predominate in green fruit tissue, β-galactosidase II is a ripening-regulated activity that increases over sevenfold during tomato fruit ripening (Pressey, 1983; Carey et al., 2001).

The tomato genome contains at least seven genes that encode β-D-galactosidase that are designated as TBG1 to TBG7 (Smith et al., 1998; Smith and Gross, 2000; Carey et al., 2001). Transcripts of all members of family are detected in tomato fruit and other plant tissues but expression of *TBG*1, 3, 4, and 5 increases during ripening. Among them the transcript abundance of *TBG1* and *TBG3* remained low and unchanged during ripening whereas the levels of TGB4 and 5 mRNAs continue to increase until the turning stage of ripening before declining. *TBG*6 mRNA exhibits highest abundance in fruit tissue but is undetectable in the ripening fruit. The nonsoftening tomato mutants *rin* and *nor*, that exhibit much reduced loss of "ripening-related" cell wall Gal compared to wild-type fruit (Gross, 1984), show absence of ripening-related rise in β-galactanase (β-galactosidase II) activity (Carey et al., 1995). These mutants show a marked reduction in TGB4 transcripts in fruits, whereas the levels of *TBG*1, 3, and 5 are similar to wild type (Moctezuma et al., 2003). Interestingly, the mRNA of *TBG*6 continues to be present in *rin* and *nor* fruits of the same chronological age as wild-type ripening fruit (Smith and Gross, 2000). The deduced amino acid sequence of *TBG*4 corresponds to the protein termed as β-galactosidase II (Smith et al., 1998). Taken together these results suggest that *TBG*4, which is ca. 100 amino acids shorter at the carboxyl terminal end than other *TBGs* and encodes a predicted polypeptide of 78 kDa, is responsible for the Gal loss from cell wall during fruit ripening (Smith and Gross, 2000).

8.9 Enzymatic solubilization of other polysaccharide components

Multiple enzymes are required to disassemble higher-order structural components of the fruit cell wall. Structural modifications in hemicellulose–cellulose domains may facilitate entry of enzymes into the ripening fruit cell wall. These enzymes include xyloglucan

endotransglycosylase (XTH, EC 2.4.1.207), the endo-β-1,4-glucanases (EGases, EC 3.2.1.4; previously known as cellulases), endo-β-mannanase (EC 3.2.1.78) (Bewley et al., 2000), β-D-xylosidases (EC 3.2.1.37) (Itai et al., 2003), and expansins. Mannan transglycosylases regulated during ripening are very recent additions to the list of candidates for roles in glycan network disassembly (Schroder et al., 2004). Ripening-associated modifications in hemicellulose-cellulose structure appear to greatly influence the accessibility of multiple enzymes to their substrates. Expansins target components of the glycan superstructure in ripening fruits, indirectly facilitating hydrolysis of the HGA substrate by one or more pectinase (Brummell et al., 1999).

Efficient distribution of C-14-starch into glucose, fructose, and sucrose revealed considerable sugar interconversions indicating active gluconeogenesis during mango fruit ripening (Yashoda et al., 2006). But the texture of Kent mango is most likely moderated by changes in solubility of insoluble pectin or by nonpectin components in the cell wall as the temperature gradient infusion of fresh-cut mangoes with PME and/or calcium chloride had no impact on hardness and adhesiveness (Banjongsinsiri et al., 2004).

Biochemical changes during ripening of capsicum are characterized as an increase in free sugar levels, an increase in in situ hydrolysis of some hemicellulose fractions (Hem a, b, and c), and a general increase in the activity of cellulase, α-mannosidase, laminarinase, polygalacturanase, galactanase, mannanase, β-galactosidase, and hemicellulase (HCe) activity on Hem b and c. But the activity of xylanase, PME, and HCe on Hem a decreased during ripening (Prabha et al., 1998). Jagadeesh et al. (2004a) observed ripening specificity for β-hexosaminidase and an interrelationship between this and α-mannosidase activities, which may be responsible for the textural softening associated with capsicum fruit ripening.

The amount of cellulose does not decline during ripening in most fruits (Maclachlan and Brady, 1994; Sakurai and Nevins, 1997), and it is presumed that depolymerization of cellulose is not a major feature of ripening. In avocado, a loss of fibrillar material from the wall occurred during ripening that was due to the depolymerization of large unbranched molecules of cellulose and an increase in the proportion of crystalline cellulose, suggesting a preferential degradation of peripheral amorphous cellulose chains (Platt-Aloia et al., 1980; O'Donoghue et al., 1994).

During peach fruit development and ripening, cell wall undergoes several structural and biochemical changes driven by several hydrolases (Bonghi et al., 1998). Among these, the endo-β-1,4-β-glucanase (EGase), or cellulase, may play a crucial role in cell wall hydrolysis. During the four stages of peach growth, EGase activity was high during S1 and early S2, declined during S3, and increased with the onset of ripening (S4), implying that the EGases is involved in early fruit growth and the initial phases of softening. The presence of two isoforms and the dual effect of propylene on enzyme activity suggest that different EGase genes operate during the early and late developmental stages in peach.

Detectable endo-β-1,4-glucanases (EGases) enzyme activity is first observed in large green fruits of strawberry, but a steep increase occurs in white fruits when the ripening process starts (Trainotti et al., 1999). This process is then accompanied by a further increase in EGase activity, which appears to be doubled in red ripe fruits.

An increase in cellulase (endo-1,4-β-D-glucanase) accompanies progressive softening, loss of skin strength, and a breakdown of cell walls in the mesocarp of raspberry, indicating its involvement in fruit separation as well as softening (Sexton et al., 1997). The initial

loss of avocado mesocarp firmness during fruit ripening may also be linked to the onset of cellulase activity (O'Donoghue et al., 1994), but no relationship between cellulase activity and softening of banana fruit was observed (Xue et al., 1995).

Pectin solubilization and release of side chain-derived galactose have been shown to be temporally coincident during early ripening in avocados and melons (Sakurai and Nevins, 1997; Rose et al., 1998). During ripening of multiple fruits, polyuronides are released from loose associations, whereas most Gal lost is derived from tightly held cell wall components enriched in rhamnogalacturonans. Therefore, in many cases, cell wall sources of solubilized pectins during ripening may not represent polymers targeted by β-galactosidase (β-Gal) (Redgwell et al., 1997b).

With advancement in ripening of papaya fruit, increased structural solubilization and concomitant depolymerization of pectin are observed (Manrique and Lajolo, 2004). A decline in the levels of galactose, galacturonic acid, and nonglucose monosaccharides indicated the association between polysaccharides from matrix and microfibrillar phases.

The β-D-galactoside galactohydrolase enzymes were capable of differentially hydrolyzing the cell wall of papaya as evidenced by increased pectin solubility, pectin depolymerization, and degradation of the alkali-soluble hemicelluloses. Hemicellulose seemed to be hydrolyzed more extensively than the pectins. The ability of the β-galactanases to markedly hydrolyze pectin and hemicellulose suggests that galactans provide a structural cross-linkage between the cell wall components (Lazan et al., 2004).

α-Galactosidase is one of the exoglycosidases capable of hydrolyzing α-1,6-linked α-galactoside residues. α-Galactosidases remove galactosyl moieties from stored galactomannan polysaccharides in germinating seeds, and can be used to change their rheological properties (Gao and Schaffer, 1999). Although α-galactosidase activity was observed to increase during ripening in tomatoes (Jagadeesh et al., 2004b), ethylene-responsive Cecona apricots and ethylene-resistant San Castrese apricot (Botondi et al., 2003), the exact role of α-galactosidase in these fruits is yet to be established.

α-Galactosidase activity increased concomitantly with firmness loss in papaya, and this increase was largely ascribed to α-gal 2 (Soh et al., 2006). The protein level of α-gal 2 was low in developing fruits and generally increased with ripening. α-Galactosidase 2 markedly increased pectin solubility and depolymerization, while the polymers were still structurally attached to the cell walls. The close correlation between texture changes, α-gal 2 activity, and protein levels as well as capability to modify intact cell walls suggest that the enzyme might contribute to papaya fruit softening during ripening.

In cellulose and the hemicellulose xyloglucan interactions, which typically comprise about two thirds of the dry wall mass, xyloglucan binds noncovalently to cellulose, coating and cross-linking adjacent cellulose microfibrils (McCann et al., 1992). The resulting extensive xyloglucan-cellulose network is thought to act as the major tension-bearing structure in the primary wall. Xyloglucan-metabolizing enzymes therefore represent a potentially important mechanism for controlling wall strength and extensibility. Cleavage of load-bearing xyloglucan cross-links by hydrolytic enzymes might be a means of achieving rapid wall loosening. Enzymes, capable of splitting and reconnecting xyloglucan molecules in rapidly growing plant tissues, were named as xyloglucan endotransglycosylase (XET) (Smith and Fry, 1991), while Nishitani and Tominaga (1992) described them as endoxyloglucan transferase (EXT, later redesignated as EXGT).

In peach cell wall, the xyloglucan, a dominant constituent of hemicellulose, gradually degraded throughout the fruit-softening period. Initially, the xyloglucan degrades during which the pectin remains insoluble, and later, pectin becomes soluble concurrently with continuous xyloglucan degradation. However, Saladie et al. (2006) suggested that xyloglucan endotransglucosylase/hydrolases (XTHs) do not represent primary cell wall–loosening agents in tomatoes.

Mannanase activity was found to increase during the later stages of ripening in tomato, but no corresponding increase in LeMAN4 mRNA was noticed, suggesting that either this gene is subjected to posttranscriptional regulation or the LeMAN4 protein remains inactive or sequestered until the later stages of ripening (Carrington et al., 2002). It may also be possible that the natural substrate of tomato fruit mannanase is not a cell wall mannan. Reduction in the mannose content of the hydrolyzed polymeric fractions of ripe mango revealed the possible involvement of an endomannase and α-mannosidase, the two major enzymes, in mango fruit softening and ripening (Yashoda et al., 2007).

The increase in endo-β-mannanase activity is greatest in the tomato skin and less in the outer and inner pericarp regions. This enzyme is probably bound to the walls of the outermost cell layers of the fruit during ripening. Endo-β-mannanase may be produced and sequestered in a mature-sized inactive form during early ripening. Most nonripening mutants of tomato exhibit reduced softening and lower endo-β-mannanase activity, but it may not be responsible for softening as some cultivars that ripen normally do not exhibit any endo-β-mannanase activity in the fruit (Bewley et al., 2000).

In olive fruit cell walls, decreases in arabinose, xylose, glucose, and uronic acid levels were observed, together with a slight increase in mannose on ripening. At the beginning of ripening, pectic polymers were the major constituents. Between the green and cherry stages of ripening, a significant loss of homogalacturonans was observed. Between the cherry and black stages of ripening, rhamnogalacturonan side chains were also released in addition to homogalacturonans (Jimenez et al., 2001).

In pears, cell wall degradation is correlated with a decrease in firmness during ripening, and the modification of both pectin and hemicellulose are essential for the development of a melting texture. Different softening behaviors during ripening among the pear fruits may be caused by different endo-PG activity and different expression of PG genes (Hiwasa et al., 2004). The increase in the activities of β-galactosidase (β-Gal) and α-L-arabinofuranosidase (α-Af) during pear ripening correlated well with a concomitant decrease in flesh firmness. The β-Gal and α-Af may not mediate difference in fruit softening between two pears, but that they could play some role(s) in cell wall changes, perhaps in cooperation with other cell wall–modifying enzymes such as PG (Mwaniki et al., 2007).

The textural changes were most noticeable at the preclimacteric stage in ripening sapote mamey fruit. The water-soluble pectin increased at a different rate than firmness decreased. No correlation between PG or PME activity and changes in firmness was observed in ripening fruits, though a low correlation was seen between β-GAL activity and softening. Fruit pulp softening was not dependent on a single enzyme activity (Arenas-Ocampo et al., 2003).

The modification of cell wall polysaccharides during softening of grape berries is a complex process involving subtle changes to different components of the wall, and in many cases only small amounts of enzyme activity are required to effect these changes (Nunan et al., 2001).

8.10 Role of expansin in regulating fruit softening

One potential regulator of pectin solubilization early in ripening is expansin. The mechanisms by which expansins operate are still unclear, but they lack significant transglycosylase or hydrolase activity (McQueen-Mason and Cosgrove, 1995). It has been hypothesized that expansins modify cell wall structure by interfering with the ability of xyloglucans or other hemicelluloses to hydrogen bond to cellulose microfibrils (Cosgrove, 1999). In fact, expansins have structural features similar to proteins that bind polysaccharides (Cosgrove, 1999). Expansin might diffuse down a cellulose chain, incrementally displacing hydrogen bonds between cellulose and interacting glycan, allowing the conversion of existing wall tension to polymer creep (Cosgrove, 2000).

Expansins are a class of proteins identified by their ability to induce the extension of isolated plant cell walls. Expansins were originally identified as cell wall–loosening proteins because of their unique ability to induce cell wall relaxation and extension in isolated cell walls, and are now generally accepted to be key regulators of wall extension during growth (Cosgrove, 2005). The expansins belong to a family of proteins that appear to be involved in the disruption of noncovalent bonds between cellulose microfibrils and cross-linking glycans. Expansin may also function to loosen the association between the structural polysaccharide in the wall by disrupting hydrogen bonding the cellulose microfibrils and the tightly associated xyloglucan polymers (McQueen-Mason and Cosgrove, 1995). Besides playing an apparently key role in wall expansion, and hence in cell growth, expansins have been implicated in an increasing number of processes during plant growth and development such as fruit ripening, pollination, germination, and abscission (McQueen-Mason and Rochange, 1999).

Expansins are encoded by an extensive multigene family and have been divided into α- and β-expansins. Two further subfamilies γ- and δ-expansins, which are truncated versions of α- and β-expansins, have been identified recently. Functional roles for γ- and δ-expansins have yet to be defined, although recent data indicate a signaling role for γ-expansins (Li et al., 2003). The expression of an expansin specifically during the stages of fruit softening and wall breakdown suggests that expansins might function in cell wall disassembly (Rose and Bennett, 1999). Brummell et al. (1999) reported that plants with overexpressed LeEXP1 had softer fruits, whereas underexpressors had firmer fruit confirming the role of expansins in tomato fruit softening. Powell et al. (2003) also demonstrated that concurrently suppressing the expression of both LeEXPI and LePG increases fruit firmness more than suppressed expression of either gene alone.

Depolymerization of polyuronides was markedly reduced in expansin-deficient plants as they became red ripe or even overripe. However, no increased depolymerization of pectins was observed in transgenic lines that overaccumulated recombinant expansin late in the ripening. Although no direct effect of expansin on the action of PG or other pectinase is reported, the confinement of obstructed pectin depolymerization to late-ripening stages was consistent with the interpretation that expansin may have indirectly limited pectin breakdown.

The expression of α-expansin gene, *MiExpA1* is correlated with softening in mango. The expression of this gene is under dual control, being triggered by ethylene treatment within 90 min followed by a ripening-associated peak in transcript accumulation on the third day after ethylene treatment. At the protein level, expression of the expansin is

detectable from the second day itself and continues throughout the course of softening. Treatment with 1-methylcyclopropene (1-MCP) inhibits both ripening/softening as well as MiExpA1 transcript and protein accumulation. It is suggested that MiErpA1 expression is ethylene dependent, and its expression increases with the progression of ripening (Sane et al., 2005).

8.11 Transgenic approaches to enhance cell wall integrity

The functions of several enzymes active in ripening fruit have been explored using transgenic strategies. Table 8.3 summarizes phenotype of some of transgenic plants underexpressing these enzymes and proteins. Antisense inhibition of PG had slight effect on fruit softening but significantly changed depolymerization of pectins resulting in increase in juice viscosity (Thakur et al., 1997). Transgenic plants expressing an antisense gene of PME showed over 95% reduction in fruit PME activity and resulted in marked improvement in juice viscosity and increased total soluble solids in fruits (Tieman et al., 1992; Gaffe et al., 1994, Thakur et al., 1996a, b). Fruit softening was not affected during normal ripening but showed breakdown of tissue integrity after extended storage (Tieman and Handa, 1994).

Homology-dependent silencing of *TBG*1 resulted in about 90% reduction in its transcript accumulation but had affected neither the total exogalactanase activity, cell wall galactose content, or fruit softening (Carey et al., 2001). These results suggest that TBG1 either does not contribute significantly to total β-galactosidase activity or has activity that is specific to a minor cell wall component. Suppression of *TBG3* by its antisense RNA gene resulted in several changes including up to 75% reduction in extractable exogalactanase activity, simultaneous reduction in *TBG*1 and *TBG*4 transcript levels, and increased cell wall galactose content. These change had little effect on ripening-related fruit softening, but fruit deteriorated slowly during long-term storage (de Silva and Verhoeyen 1998). Juice from these transgenic fruits showed increase in insoluble solids and viscosity. Transgenic tomato fruits expressing about 1,500 bp of TGB4 in antisense orientation showed strong reduction in TGB4 transcripts and about 90% reduction in extractable exogalactanase activity (Smith et al., 2002). Compared to wild type free galactose levels in all TBG4 antisense lines were lower at mature green stage 4, but in ripening fruits. Also, the total fruit cell wall galactosyl contents were not affected by the antisense gene. All of the antisense lines had reduced free galactose levels at mature green stage 4, but levels comparable with control during ripening. Total cell wall galactosyl contents in the antisense fruit were not significantly different from control fruit. Fruits from several independent transgenic were firmer than control. Fruits from the transgenic line, designated 1-1, exhibiting maximum reduction in TBG4 transcripts and exogalactanase activity, and the highest galactosyl residue content during early stage of ripening were 40% firmer than control (Smith et al., 2002). By expressing *TBG4* in yeast, Smith and Gross (2000) have confirmed that it encodes both a galactosidase and an exogalactanase. Taken together above results provide a strong evidence for the involvement of β-galactosidase in cell wall modification leading to fruit softening.

Results with other genes have indicated that specific members of the β-galactosidase, expansin, and PL gene families partially regulate softening in tomatoes or strawberry (Brummell et al., 1999; Jiménez-Bermúdez et al., 2002; Smith et al., 2002). However, functional analyses of specific PGs, PMEs, XTHs, and EGases in tomatoes, strawberries, and peppers

Table 8.3 Transgenic genetics to determine role cell wall–modifying enzymes

Enzyme/protein glycanases	Transgene	Fruit	Phenotype	References
Polygalacturonase (PG)				
PG	Overexpression	Tomato	No effect	
			Increased pectin solubilization	Giovannoni et al. (1989)
	Antisense	Tomato	Slightly firmer	Langley et al. (1994)
			Increased juice viscosity	Schuch et al. (1991)
			Reduced pectin solubilization	Kramer et al. (1992)
				Brummell and Labavitch (1997)
PG-β-subunit	Antisense	Tomato	Increases fruit softening	Watson et al. (1994)
			Deceased middle lamella cohesion	Chun and Huber (2000)
			Reduced tissue integrity	
Pectin methylesterase (PME)				
PME	Antisense	Tomato	No effect on softening	Tieman et al. (1992)
			Reduced tissue integrity at late-ripening stages	Hall et al. (1993)
			Increased degree of methylesterification	Tieman and Handa (1994)
			Reduced pectin degradation	Thakur et al. (1996a, b)
			Increased soluble solids	
			Increased juice and paste viscosity	
			Increased serum viscosity	
Pectate lyase	Antisense	Strawberry	Increased fruit firmness	Jiménez-Bermúdez et al. (2002)
Cellulase				
Endo-1,4-β-glucanase	Antisense	Pepper	No reduction xyloglucan depolymerization	Harpster et al. (2002a)
	Overexpression	Pepper	No increase in xyloglucan depolymerization	Harpster et al. (2002b)
β-Galactosidase				
β-Galactosidase 1	Antisense	Tomato	No effect on cell wall galactose	Carey et al. (2001)
			No effect on fruit softening	
β-Galactosidase 3	Antisense	Tomato	Reduction in *TBG*1 and *TBG*4 transcripts	de Silva and Verhoeyen (1998)
			Higher GalA in cell wall	
			Reduced fruit deterioration	
β-Galactosidase 4	Antisense	Tomato	Reduced fruit softening	Smith et al. (2002)
			Higher GalA in cell wall	
Expensin	Antisense	Tomato	Increased firmness	Brummell et al. (1999)
			Reduced polymer	
Expansin and PG	Both antisense	Tomato	Increased fruit firmness	
			Modify juice rheology	Kalamaki et al. (2003)
PG and PME	Both antisense	Tomato	Modify juice rheology	Errington et al. (1998)

have failed to establish their roles in softening (Lashbrook et al., 1998; Brummell et al., 1999; Woolley et al., 2001; Harpster et al., 2002a, b). Transgenic fruit firmness was not significantly altered when PG or expansin action was suppressed individually, but increased when genes encoding both proteins were simultaneously downregulated (Powell et al., 2003).

8.12 Postharvest factors affecting structural deterioration

The major postharvest problem with storage of fruits and vegetables is the excessive softening. Ripening of many fruits is mainly orchestrated by biosynthesis of ethylene that triggers a serial biochemical and physiological process inducing the softening in texture. In case of *Capsicum annuum* fruits, softening during ripening is associated with alteration in pericarp cell wall and the breakdown of middle lamella pectins (Sethu et al., 1996).

8.12.1 Processing

In olives, the lye treatment and wash causes an exchange of arabinans from carbonate-soluble and 4 M KOH-soluble fractions to the water-soluble fraction. The main change in pectins was a movement of homo- and rhamnogalacturonans from water-soluble and carbonate-soluble fractions to the imidazole-soluble fraction, but a partial solubilization of alkali-soluble and cellulose-linked pectins during lye treatment, wash, and fermentation was also observed (Jimenez et al., 1998).

California Black Ripe processing of olives was accompanied by general solubilization of polysaccharides, and pectins and a noncellulosic glucan component were most clearly affected. Soluble polysaccharides accumulated in processing liquids. Analysis of polysaccharides extracted from cell walls suggests that the polymer most extensively solubilized and eluted during processing is relatively unbranched pectin (Araujo et al., 1994).

In mechanically injured tissues (fresh-cut) of papaya the PG, cellulase and β-GAL activities increased within 24 h of cutting and remained significantly higher during storage as compared to intact fruits. These enzyme activities were accompanied by an increase in both 1-aminocyclopropane-1-carboxylate synthase **(**ACS) and 1-aminocyclopropane-l-carboxylate (ACC) activities raising the possibilities of enhanced ethylene production, thereby stimulating ripening (Karakurt and Huber 2003). In tomatoes, wounding resulted in reduction or complete cessation of PG synthesis (Chung et al., 2006). The increase in PG activity during ripening is due to de novo synthesis (Tucker et al., 1980; Bird et al., 1988; Biggs and Handa, 1989), and reduction in PG gene expression was observed after wounding. Chung et al. (2006) also reported that wounding might also impair the ability of ripening tomato tissues to recover PE activity and β-galactanase activity.

Carbohydrate analysis of partially defatted almond seeds revealed important changes in cell wall polysaccharides. At low extraction percentages (up to 33%), pectic polysaccharides and hemicellulosic xyloglucans were the main type of polymers affected, suggesting the modification of the cell wall matrix, although without breakage of the walls. At higher extraction rates (up to 64%), a major disruption of the cell wall occurred as indicated by the losses of all major types of cell wall polysaccharides, including cellulose. At higher extraction rates, fatty acid chains are able to exit the cells either through unbroken walls, or the modification of the pectin-hemicellulose network might have increased the porosity of

the wall. Due to high pressure, a progressive breakage of the cell walls was observed, which allows the free transfer of the fatty acid chains from inside the cells (Femenia et al., 2001).

8.12.2 Heat

Postharvest heat treatments lead to an alteration of gene expression, and fruit ripening can sometimes be either delayed or disrupted. Cell wall–degrading enzymes and ethylene production are frequently the most disrupted, and their appearance is delayed following heating. Fruit sensitivity to heat treatments is modified by preharvest weather conditions, cultivar, rate of heating, and subsequent storage conditions (Paull and Chen, 2000). Prestorage heat treatment appears to be a promising method of postharvest control of decay. Heat treatments against pathogens may be applied to fresh harvested commodities by hot water dips, by vapor heat, by hot dry air, or by a very short hot water rinse and brushing.

Banana fruit exposed to elevated temperatures showed increased softening, and softening was inhibited by 1-MCP treatment, which suggests that heat enhances synthesis of new ethylene sites which mediated banana fruit softening (Jiang et al., 2002). In case of apple slices, the heat treatment inhibited slice texture changes but reduced sensory firmness (Bai et al., 2004).

In strawberries, heat treatments reduced EGase and β-Xyl activity and delayed hemicellulose degradation. The application of heat treatment affected the solubization of pectins and hemicelluloses. PG and β-Gal activity was also inhibited by heat treatment, but PME activity was enhanced (Vicente et al., 2005).

PG and xylanase decreased in papaya by hot water treatment (49°C for 120 min), and dissolution of the middle lamella and destruction of the cell wall were inhibited (Bacay-Roldan and Serrano, 2005). PG, which was altered by vapor heat treatment, influenced the development of physiological injury in papaya (Suzuki et al., 1994).

Heat treatment inhibits the synthesis of cell wall hydrolytic enzymes (Schirra et al., 2000). Prestorage heat treatment could delay the ripening of “Gala” and “Golden Delicious” apples and maintain storage quality (Shao et al., 2007). Heating “Golden Delicious” apples for 4 days at 38°C reduced decay and maintained fruit firmness during 6 months of storage at 0°C (Sams et al., 1993).

Cooking resulted in an increase in the water-soluble pectins and a decrease in the pectins associated with cellulose. The total cell wall polysaccharide and galactose content of the squash cultivars remained unchanged for up to 2 months of storage and decreased later (Ratnayake et al., 2003).

8.12.3 Physiological disorders

Symptoms of water soaking in watermelon were accompanied by increases in the levels of water- and CDTA-soluble polyuronides and significant molecular mass downshifts in polyuronides in both immature and ripe fruit (Karakurt and Huber, 2002). Catabolic reactions targeting the cell walls contribute to the development of water-soaking disorder in watermelon, and PG, EXP, LOX, PLC, and PLD levels increase with its onset and development (Karakurt and Huber, 2004).

Depolymerization of high-molecular-mass *trans*-1,2-CDTA-soluble pectin occurred in severely watercored pear fruits, but there were no such metabolic changes in the

Na_2CO_3-soluble fractions. The activities of β-galactosidase and β-glucosidase increased as the zone of watercore expanded. Catabolism of carboxymethyl cellulose by cellulase was not different between sound and watercored tissues, but the activity against xyloglucan was higher in watercored tissue than the sound tissue adjacent to the watercore zones. Structural changes in sound and severely watercored tissue showed cell separations with the accumulation of pectic materials in the intercellular space of severely watercored tissues (Chun et al., 2003).

Water soaking, a physiological disorder characterized by a glassy texture of the flesh melon fruit, is due to increased water mobility. Alteration of the cell wall and the presence of large intercellular spaces were correlated with a severe depletion of cell wall calcium. Water soaking developed during the late stages of fruit ripening. The major changes were observed in a protein implicated in calcium signaling processes. While the amount of total calmodulin, the ubiquitous calcium-binding protein, was not modified, a particular calmodulin-binding protein (CaM-BP) was absent in water-soaked but not in sound mature tissues. This CaM-BP may be a marker or a determinant of this physiological disorder (du Chatenet et al., 2000).

Gel breakdown in inner mesocarp tissue of plums was associated with high viscosities of water-soluble pectin with low levels of extractable juice. In outer mesocarp tissue where extractable juice levels were higher, overripeness developed. Cell walls of inner tissue were thicker and had a better developed middle lamella than outer tissue. Inner mesocarp tissue was composed of larger cells than outer tissue (Taylor et al., 1993).

8.12.4 Chilling and freezing injury

Insoluble pectin levels declined during ripening and cold storage of plum fruit with a concomitant increase in soluble pectin levels. Neither harvest maturity nor storage time had a significant effect on the concentration of calcium pectate, and this pectic fraction did not appear to influence development of gel breakdown (GB). Water-soluble pectin and availability of cell fluids indicated a high gel potential in plums. Significant levels of GB developed only in plums harvested at postoptimum maturity. In GB fruit, higher sugar levels and loss of cell membrane integrity probably enhanced formation of pectin sugar gels as cell fluids bind with pectins in cell walls (Taylor et al., 1995).

Four β-D-galactosidases (GA-ase I, II, III, and IV) and one α-L-arabinofuranosidase (AF-ase) activities were detected in cell wall extracts from apples stored at 0°C for 5 months (Yoshioka et al., 1995). The GA-ase degrades polyuronides and releases galactose. GA-ase II, III, and IV fractions contained arabinogalactan-degrading and galactose-releasing activities, but GA-ase I failed to degrade arabinogalactan. AF-ase fraction degraded polyuronide and arabinogalactan releasing arabinose and other sugars. The activities of GA-ase II, III, and IV decreased gradually as the apples softened in storage. The activities of GA-ase I and AF-ase, which were not detectable at harvest, increased during storage. These galactose- and arabinose-releasing activities may be involved in the degradation and solubilization of polyuronide, araban, and galactan in the cell walls of apples during softening.

Postharvest life in peach is limited by chilling injury. The initial response to low temperature is considered to involve physical factors such as membrane alteration and protein/enzyme diffusion, but physiological changes that lead to losses of structural integrity and overall fruit quality also occur (Morris, 1982). During softening, dissolution of the

ordered arrangement of cell wall and middle lamella polysaccharides occur. As the fruit ripens, a substantial portion of its cell wall pectins are converted to a water-soluble form affecting the texture (Labavitch, 1981).

The delay in ripening of cold-stored peaches has been associated with reduced ability of fruits to convert insoluble pectic substances into soluble pectin (Lurie et al., 1994), and with the inhibition of PG activity (Artes et al., 1996). The major changes involved in softening and chilling injury in peaches are the catabolism of cell walls and the development of an intercellular matrix containing pectins (Harker and Maindonald, 1994). Gel-like structure formation in the cell wall due to the deesterification of pectins without depolymerization leads to the development of woolliness in peach (Lurie et al., 2003).

Peach fruits develop mealiness (pastiness), which is associated with separation of middle lamella without extensive degradation of cell wall. Mealiness has been attributed to the presence of insoluble low methoxyl pectic substances of high molecular weight that are formed by the action of PE during chilling. The affected cells showed larger primary walls separated, forming a continuous extracellular matrix. The intracellular spaces were characterized by the presence of amorphous pectic substances and polysaccharides. At the ultrastructural level, dissolution of the middle lamella, cell separation, irregular thickening of the primary wall, and plasmolysis of the mesocarp parenchyma cells were seen as internal breakdown progressed (Luza et al., 1992).

Uronic acid content was higher in both water-soluble and -insoluble pectin fractions in sound peach fruit compared to fruit with internal breakdown symptoms. The chilling-injured fruits were characterized by 26% higher content in total neutral sugars compared to sound fruit, which was mainly attributed to increased galactose, arabinose, and glucose contents, whereas tissue derived from sound fruit had a 27% higher cellulose content compared to chilling-injured tissue. Decreased activities of both PG and PME, accompanied by decreased levels of cation binding in the cell walls, primarily of calcium, were recorded in the brown-fleshed tissue (Manganaris et al., 2006).

Ruoyi et al. (2005) showed that combination of chitosan coating, calcium chloride, and intermittent warming partially inhibited PG activity, slowed down the increase in soluble pectinefic substances. Addition of calcium chloride and intermittent warming could keep the intactness of cell wall and reduce fruit sensitivity to injury in peach.

Endo-PG, PE, and endoglucanase (EGase) activities of delayed-storage nectarines fruit were same as the control fruit at the beginning of storage, although exo-PG was higher. Endo-PG activity was lower in control than delayed-storage fruit at the end of storage, while PE activity was higher, and exo-PG and EGase activities were similar. Prevention of chilling injury by delayed storage (DS) appears to be due to the ability of the fruit to continue progressive and slow cell wall degradation in storage, which allows normal ripening to proceed when the fruits are rewarmed (Zhou et al., 2000).

8.12.5 Modified atmosphere

Fruit softening is associated with the disassembly of primary cell wall and middle lamella structure. The changes in cell wall structure and composition result from the composite action of hydrolytic enzymes produced by fruits, which include PG, PE, PL, β-GAL, and cellulases (Brummell and Harpster, 2001). High-oxygen atmosphere retards the decrease in firmness in grapes (Deng et al., 2005), sweet cherries (Tian et al., 2004), fresh-cut carrots

(Amanatidou et al., 2000), and strawberries (Wszelaki and Mitcham, 2000). Deng et al. (2005) observed that decrease in firmness of grapes under different oxygen storage was accompanied by a dramatic decrease in hemicellulose and moderate decrease in cellulose and total pectins, which indicates that the softening in grapes is due to increased depolymerization and degradation of cell wall polysaccharides. At higher oxygen storage, grapes maintained firmness, which coincided with higher retention of cell wall polysaccharides. The lower levels of water-soluble pectins in high-oxygen atmosphere were correlated with delayed softening, and the activities of PG, PE, β-GAL increased to lower extent than air storage, which indicates that higher oxygen might have inhibited relative enzyme activities, reducing the degradation and depolymerization of pectin substances.

Cellulase activity in grapes increased slightly over time, and its activity was slightly lower in high oxygen compare to air (Deng et al., 2005). In controlled atmosphere (CA)-stored apples, ripening-related softening was inhibited after an initial loss of firmness. However, softening resumed after transfer of apples to normal atmosphere storage at 8°C (Ingham et al., 1998).

The retardation of carambola fruit softening by MAP and/or LT, which correlates closely with delayed solubilization and depolymerization of the chelator-soluble polyuronides, may partly be attributed to suppression of the increase in activity of the major wall hydrolases. Suppression of the enzyme activities in fruit under MAP also appears to contribute to increased tolerance of the carambolas to CI incidence (Ali et al., 2004).

Delayed (2 days at 20°C before storage) and controlled atmosphere (fruits stored at 10% CO_2, 3% O_2) storage of nectarines fruits prevented wooliness during ripening of fruits after 4- to 6-week storage at 0°C compared to fruits stored immediately in 0°C, 95% RH air (Zhou et al., 2000). At the time of removal, the delayed stored fruits exhibited similar levels of PG and PE transcripts but higher PG and lower PE activities compared to control fruits. CA-repressed transcript levels of both PG and PE and activity of PG, but the PG activity recovered during the 7-day ripening period. Whereas the endoglucanase activity declined during ripening in all fruits, control fruits retained more activity than DS or CA fruits. On the basis of these results, Zhou et al. (2000) have suggested that the ratio between PG/PE at the time of removal of the delayed stored fruits or during ripening of CA stored fruits plays a significant role in the development of fruit wooliness.

A hydrophobic coating formulated with maltodextrins, carboxymethylcellulose, propyleneglycol, and a mixture of sorbitan esters was applied to preclimacteric "Manila" mangoes. The fruit treated with the coating suffered less mesocarp softening along with concomitant reductions of PG and cellulase (Cx) activities than did control fruit. After the initial storage period, activity of PG increased steadily during further ripening of coated fruit (Diazsobac et al., 1997).

Coating citrus with low-molecular-weight chitosan (LMWC, Mw = 15 kDa) improved firmness and exhibited greater antifungal resistance than 2-(4-thiazolyl)benzimidazole (TBZ), and its quality was maintained for longer (Chien et al., 2007).

8.12.6 Pathogen attack

In apple and tomato fruits, *Penicillium expansum* infection caused reduction in the molecular mass of hemicelluloses, particularly in the xyloglucan. Xyloglucan endotransglucosylase/hydrolase (XTH)-specific activity decreased drastically during the infection process

in both fruits. XTH reduction during the infection might be related with the fungus attack mechanism. Decrease in activity and the consequent lower xyloglucan endotransglucosylation, together with the increase in endoglucanases, would permit fungal access to the cellulose-xyloglucan network, increase the efficiency of cellulose hydrolysis, and thus facilitate the progress of the fungal infection. Hemicellulose degradation is important in the breakdown of plant cell walls, causing cell wall loosening, increasing the porosity of the wall, and allowing the colonization of plant tissue (Miedes and Lorences, 2004).

In bell pepper fruit tissue, massive fungal colonization was followed by extensive degradation of the pectin component of host walls and middle lamella due to the necrotrophic growth of *Botrytis cinerea*. Cellulose breakdown was limited to small wall areas. The disruption of host walls and the reduction of pectin labeling appeared to parallel levels of cell wall–macerating enzymes isolated from *B. cinerea*-infected tissue. High levels of PG and trace amounts of cellulase were detected in *B. cinerea*-infected tissue. In chitosan-treated tissue, the preservation of pectin-binding sites and the intense and regular cellulose distribution over host walls suggested that chitosan might have prevented the maceration of host tissue by *B. cinerea*. Chitosan not only was effective in reducing the production of PGs by *B. cinerea*, but also caused severe cytological damage to invading hyphae, which may be responsible for the limited ability of the pathogen to colonize tissues in the presence of chitosan (Elghaouth et al., 1997).

Pear PG inhibitor protein (PGIP) caused partial inhibition of the crude mixture of Botrytis enzymes and increased the ratio of dimeric to monomeric uronide products. However, no accumulation of larger oligomeric breakdown intermediates was detected, and no impact on ethylene elicitor activity of the digestion products was observed. Differential inhibition of the *B. cinerea* PG isozymes by pear PGIP was observed (Sharrock and Labavitch, 1994).

8.12.7 Irradiation

The biological effect of gamma rays is based on the interaction with atoms or molecules in the cell, particularly water, to produce free radicals, which can damage different important compounds of plant cell. The UV-B/C photons have enough energy to destroy chemical bounds, causing a photochemical reaction. Gamma rays accelerate the softening of fruits, causing the breakdown of middle lamella in cell wall. They also influence the plastid development and function, such as starch–sugar interconversion. The penetration of UV-B light into the cell is limited, while gamma rays penetrate through the cells. For this reason, UV-B light has a strong effect on surface or near-to-surface area in plant cells. Plant pigments, such as carotenoids and flavonoids, save plant cells against UV-B and gamma irradiation (Kovacs and Keresztes, 2002).

UV light has been used as a postharvest treatment to enhance shelf life of various fruits and vegetables (Liu et al., 1993; Maharaj et al., 1993). The beneficial doses of UV-C are reported to induce the accumulation of phytoalexins (Devlin and Gustine, 1992) and activate genes, encoding pathogenesis-related proteins (Green and Fluhr, 1995). Barka et al. (2000) demonstrated that UV-C treatment resulted in a reduction in softening and the lowered activities of cell wall–degrading enzymes (PG, PME, cellulase, xylanase, β-D-galactosidase and protease) in tomato fruit. They proposed that cell wall–degrading enzymes are one of the targets of UV-C irradiation by inducing their proteolysis or the reduction of their

de novo synthesis, and it contributed to a delay of the cell wall degradation and prevented softening.

Irradiation induces softening in mature green and pink tomato fruits within hours following irradiation, and differences between irradiated and control fruit persisted throughout postirradiation storage. Fruit irradiated at the mature green stage softened during postirradiation storage but exhibited an apparently irreversible suppression in PG activity, with levels remaining $\leq$10% of those of nonirradiated fruit. PG activity was less strongly affected in irradiated pink fruit than in mature green fruit, but activity remained reduced relative to the controls. PME and β-galactosidase activities were significantly enhanced in irradiated fruit of both ripening stages in the early period following irradiation, but reductions were noted after prolonged storage (Elassi et al., 1997).

The firmness of irradiated papaya fruits (500 Gy) was retained at least 2 days longer than in control fruits and also had a slower rate of softening. The activity patterns of PG, PME, and β-galactosidase were related to pulp softening and were affected by irradiation. Irradiation may not have any direct effect on firmness, but it acted by altering the ripening-induced synthesis of cell wall enzymes, mainly PME (D'Innocenzo and Lajolo, 2001).

Fruit treated with UV-C remained firmer and softened more slowly than the control and those treated with longer durations of exposure (Gonzalez-Aguilar et al., 2004). Exposing packaged watermelons cubes to UV-C not only causes reduction in microbial populations but also extends products shelf life without affecting juice leakage and overall visual quality (Fonseca and Rushing, 2006).

Prestorage exposure of peaches with UV-C irradiation significantly reduced chilling injury. A higher accumulation of spermidine and spermine was found in peaches after UV exposure, and it is postulated that these higher levels of polyamines apparently are a response to the UV-C irradiation and might be beneficial in increasing the resistance of fruit tissue to deterioration and chilling injury (Gonzalez-Aguilar et al., 2004).

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Chapter 9

Structural Deterioration in Produce: Phospholipase D, Membrane Deterioration, and Senescence

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9.1 Introduction

Ripening and senescence are the ultimate phases in the developmental events of fruits that result in the expression of the quality characteristics inherent to the fruit. Although it is difficult to demarcate a dividing point between ripening and senescence, the former can be designated as the quality-developing phase, whereas the latter predominantly involves the loss of quality. Degradation of structural elements such as the cell wall and the plasma membrane is an inherent feature of ripening and senescence. Wilting of flowers also involves similar deteriorative changes. Harvested vegetables, when exposed to abiotic stresses such as heat, cold, and water deficit, undergo deteriorative changes in the cell membrane that result in the loss of quality. Seasonal changes that result in a decrease in light duration and intensity, temperature, etc., are natural cues for the initiation of ripening in fruits. Often such changes are associated with the biosynthesis of ethylene, which is also an initiator of ripening in many fruits and senescence in leaves. At the ultrastructural level, the events that occur during ripening and senescence reflect the deterioration of cellular structures, and in particular the cell membrane, which results in a loss of compartmentalization of ions and metabolites, leading to the loss of tissue structure and ultimately homeostasis.

The importance of regulating the deteriorative events that occur during ripening and senescence has been well recognized. Consequently, several scientific approaches and technologies have been developed with the objective of enhancing the shelf life and quality of harvested fruits, vegetables, and flowers. Some of these include attempts to stabilize the cell wall by the application of calcium, biotechnological approaches to inhibit cell wall–degrading enzymes, and modification of the ethylene biosynthetic pathway through inhibition of key enzymes (e.g., inhibition of ACC synthase by the application of aminoethoxyvinylglycine (AVG), commercially available as "ReTain" (Valent Biosciences)), and biotechnological approaches (e.g., antisense transformation of apple, tomato, and melon for ACC synthase or ACC oxidase). Inhibiting ethylene action through the application of the ethylene antagonist 1-methylcyclopropene (*SmartFresh*, AgroFresh, Inc.) has become a practice of both basic biological and practical interest. Application of 1-MCP has been successfully used to extend the shelf life of fruits (e.g., apples and pears) and flowers. The cell membrane is a key site where senescence-dependent changes are observed to occur earlier than most other

events. Continued deterioration of the cell membrane eventually leads to the loss of compartmentalization within the cell. Although we have detailed knowledge of the deleterious structural changes in the cell membrane and the associated loss of functional properties, very little attention has been given to developing strategies for the preservation of membrane structure during ripening and senescence, until recently (Ryu et al., 1997; Paliyath et al., 2003; Whitaker and Lester, 2006).

9.2 Physicochemical changes in cell membrane structure and properties

Cell membranes are dynamic entities. Both the protein and lipid components are constantly being turned over to maintain a functional state suited to the physiological state of the produce. The biochemical composition, the degree of unsaturation of acyl chains, polar head groups, and the pH of the medium are all factors that influence and regulate the functional properties of membranes and the activities of embedded enzymes. The membrane properties are precisely regulated to maintain cellular homeostasis.

The lipid composition of cell membranes can be quite heterogeneous (Yoshida and Uemura, 1986; Larsson et al., 1990). In both plasma membrane and tonoplast, phospholipids, sterols, and ceramide monohexosides were shown to be the major classes of lipids. The plasma membrane contained relatively higher levels of sterols than the tonoplast, and thus possessed a higher sterol/phospholipid ratio than the vacuolar membrane. Among the phospholipids, phosphatidylcholine and phosphatidylethanolamine were the major components, with smaller amounts of phosphatidylinositol, phosphatidylglycerol, and phosphatidylserine. Considerable changes occur during the ripening/senescence process that lead to alteration in the biophysical properties of membranes, including a transition from predominantly liquid crystalline to gel-phase lipid, a decrease in bulk lipid fluidity or an increase in microviscosity, an increase in phase transition temperature, and the formation of nonbilayer lipid structures (Thompson et al., 1987). Such changes occur as a result of the enzymatic catabolism of phospholipids and the accumulation of degradation products such as phosphatidic acid (PA), diacylglycerols, free fatty acids, and their oxidation products.

In banana, the total lipid content remained unchanged during the respiratory climacteric induced by the application of ethylene. The relative proportions of phospholipids, glycolipids, and neutral lipids remained constant during this period. However, in the phospholipid fraction, there were considerable changes in fatty acyl unsaturation and composition. The content of linolenic acid in the phospholipid fraction increased with a concomitant decrease in the linoleic acid content, thus resulting in a higher level of unsaturation (Wade and Bishop, 1978). In ripening apple fruit, the microviscosity increased from 3.46 poise in early climacteric to 4.56 poise in postclimacteric stage. The phospholipid content showed a slight increase from 6.77 to 8.75 μmol/50 g fresh weight during the same time period. However, the sterol level increased during the postclimacteric stage, resulting in a higher sterol/phospholipid ratio. The fatty acyl composition also showed changes, with a decline in unsaturated (18:3, 18:2) and an increase in saturated (16:0, 18:0) fatty acids. These changes were also associated with increased leakage of potassium ions from the apple tissue (Lurie and Ben-Arie, 1983). A decline in total phospholipid content has been observed in carnation flower petals during senescence (Fobel et al., 1987; Sylvestre et al., 1989) and in cherry tomato (Güçlü et al., 1989) and tomato (Whitaker, 1994) fruits during ripening. The decline

in total phospholipids in ripening tomato fruit pericarp tissue was associated with increases in the levels of phosphatidic acid and free fatty acids, and in the sterol/phospholipid and glucocerebroside/phospholipid ratios (Whitaker, 1991, 1992, 1993, 1994). There were also marked ripening-specific changes in sterol lipid content and composition, including an increase in total sterols, higher proportions of sterol esters, free sterols, and sterol glycosides relative to acylated sterol glycosides, and a dramatic increase in the stigmasterol/sitosterol ratio (Whitaker, 1988, 1994).

Surprisingly, there was no loss of phospholipids during chilling or after subsequent warming of mature green tomato fruit (Whitaker, 1991, 1992, 1994; Bergevin et al., 1993), but profound effects on sterol lipid metabolism were observed. Most notably, a large increase in the proportion of free sterols occurred during chilling, whereas after warming of chilled fruit there was a rapid return to at-harvest levels of free and conjugated sterol classes plus a sharp increase in the stigmasterol/sitosterol ratio (Whitaker, 1991, 1993, 1994). Palta et al. (1993) have also reported differences in phospholipid metabolism in leaves of freezing-tolerant wild-type potato species and freezing-susceptible cultivated species. Cold acclimation of both the species resulted in similar changes in plasma membrane lipids that included a decrease in palmitic acid (16:0), an increase in unsaturated to saturated fatty acid ratio, an increase in free sterol levels, especially sitosterol, and a small decrease in cerebrosides. Lipid changes specific to the freezing-tolerant species included an increase in phosphatidylethanolamine, a decrease in sterols, an increase in linoleic acid with a decrease in linolenic acid, and an increase in the acylated sterol glycoside to sterol glycoside ratio.

The lipid compositions of chloroplasts and mitochondria are quite different from that of the plasma membrane (Schwertner and Biale, 1973). Phospholipids amounted to 50–56% of total lipids in mitochondrial preparations from avocado fruits, cauliflower buds, and potato tubers. As in most cell membranes, the major phospholipids were phosphatidylcholine and phosphatidylethanolamine, but the unusual phospholipid cardiolipin (diphosphatidylglycerol) was also abundant. The glycolipids included monogalactosyl and digalactosyl diacylglycerols. Potato mitochondria had a relatively low content of phospholipids. In contrast to mitochondria, chloroplasts are highly enriched in the monogalactosyl and digalactosyl diacylglycerols relative to phospholipids, and phosphatidylglycerol is the major phospholipid. Irrespective of their composition, all membranous structures undergo enzymatic and active oxygen-mediated catabolism during senescence.

9.3 Membrane lipid catabolism during senescence

The pathway of membrane lipid degradation that occurs during ripening or senescence was delineated using several systems that included bean cotyledons (Paliyath and Thompson, 1987), carnation flower petals (Paliyath et al., 1987), broccoli florets (Deschene et al., 1991), and tomato fruit (Todd et al., 1990; McCormac et al., 1993). In most of these studies, a microsomal membrane fraction comprising the plasma membrane, endoplasmic reticulum, and tonoplast membranes was isolated by differential centrifugation of the tissue homogenate. Microsomal membranes were incubated in an enzyme assay mixture containing a radiolabeled phospholipid substrate consisting of uniformly labeled phosphatidylcholine (PC), a specific molecular species of PC (e.g., 16:0/16:0, 16:0/18:2, and 18:0/20:4), or other phospholipid classes. After a period of incubation, the reaction was terminated by acidification and the addition of chloroform/methanol (2:1 v/v), effecting a phase separation. The heavier

chloroform phase containing all the lipids, including the unreacted radiolabeled substrate and the reaction products was removed, the chloroform evaporated in a stream of nitrogen, and the concentrated lipid fraction subjected to thin-layer chromatography. The plates were developed to separate the polar lipids such as the phospholipids, and the neutral lipids such as diacylglycerols and free fatty acids. By quantifying the radiolabel in the defined lipid classes (as confirmed by authentic standards), the amount of the substrate remaining and the products formed were determined. Such experiments confirmed that the phospholipid is broken down immediately to yield PA via removal of the polar head group (choline, ethanolamine, etc.). PA rarely accumulated in the assay mixture, but appeared to be rapidly broken down to diacylglycerols through the action of phosphatidate phosphatase. Diacylglycerols were further catabolized to free fatty acids by the action of lipolytic acyl hydrolase (LAH). LAH does not have positional specificity and can remove fatty acids from either the *sn*-1 or *sn*-2 position. If the fatty acids have a 1,4-pentadiene structure (as in linoleic and linolenic acids), they may be acted upon by lipoxygenase, yielding hydroperoxide products as well as smaller-chain fatty acids and aldehydes. Accumulation of such degradation products in the membrane causes destabilization through the formation of gel-phase lipid, lipid microvesicles, and nonbilayer lipid structures.

The catabolism of radiolabeled phosphatidylcholine by broccoli microsomal membranes is shown in Fig. 9.1. The broccoli heads were stored at 10°C, and microsomal membranes

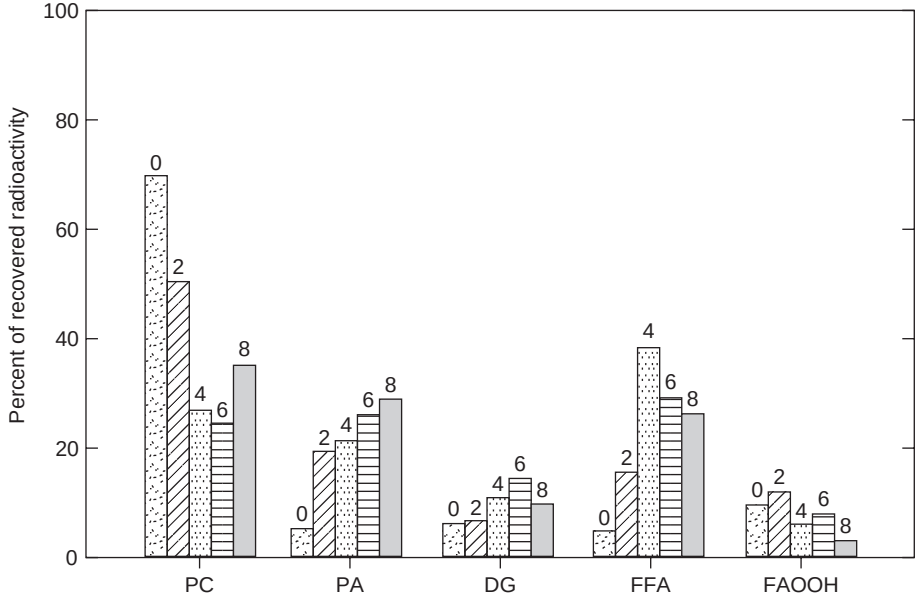


Fig. 9.1 Changes in the formation of phospholipid degradation products by microsomal membranes from florets isolated at various stages during storage of broccoli. The broccoli heads were stored in air at 10°C and a relative humidity of 75–85%. Phospholipid degradation activity was measured by incubating the isolated membranes with radiolabeled 18:0/20:4 phosphatidylcholine and quantifying the levels radiolabeled degradation products after 1.5 h of incubation. The values are expressed as percentage of recovered radioactivity. The numbers above the bars refer to days of postharvest storage. PC, phosphatidylcholine; PA, phosphatidic acid; DG, diacylglycerol; FFA, free (unesterified) fatty acids; FAOOH, peroxidized free fatty acids. (Reproduced with permission from Deschene et al., 1991.)

isolated from the florets prior to and after storing for 2, 4, 6, and 8 days. The membrane was incubated with radiolabeled PC, and catabolites in each fraction were determined. It can be seen that initially (0 day) the formation of PA, indicative of phospholipase D activity, was quite low. However, with prolonged storage, the PA level increased, followed by increases in the formation of diacylglycerols (DG), free fatty acids (FFA), and hydroperoxide products of free fatty acids (FAOOH). Broccoli is very difficult to store, and often over 50% of the produce is damaged during shipping. Broccoli is immediately washed and cooled with an ice–water mixture after harvest and is generally shipped with ice for long distances. However, by employing controlled atmosphere storage (5% CO_2, 3% O_2, 92% N_2 at 5°C and a relative humidity of 80%), the storage life of broccoli can be extended for up to 2 months (Deschene et al., 1991). These studies reflect the changes in lipid content observed in vivo during senescence including a decline in phospholipid content and an increase in neutral lipids.

The accumulation of phospholipid degradation products in the membrane can affect its biophysical properties. Membrane lipids adopt liquid crystalline, gel, micellar, and hexagonal phases based on the composition, hydration, and presence of ions (Lafleur et al., 1990). Accumulation of lipid degradation products in the membrane can cause localized transformation of membrane lipid bilayer into destabilized bilayer and nonbilayer lipid structures such as the micellar and hexagonal-phase lipid. Lipid catabolites such as alkanes, fatty alcohols, and fatty acids can alter the physicochemical properties of the lipid bilayer and, thereby, the functional state of a biological membrane (Lohner, 1991). Alkanes can induce the formation of nonbilayer structures in the membrane. Diacylglycerols induce different forms of perturbations in the bilayer depending on whether they possess saturated or unsaturated fatty acids. Saturated diacylglycerols such as dipalmitin and distearin induce lateral-phase separation of the lipids into diacylglycerol-enriched, gel-like domains and relatively diacylglycerol-free regions in the liquid crystalline phase (DeBoeck and Zidowski, 1989). Diacylglycerols are also known to cause formation of micelles and vesicles in the membrane (Allan et al., 1976). The accumulation of free fatty acids and their metabolites can induce formation of gel-phase lipid (Katsaras and Stinson, 1990). As well, a decline in phospholipid content and a relative enrichment in sterols can cause the formation of gel phase and a decrease in bulk lipid fluidity (Duxbury et al., 1991). The formation of gel-phase lipid during senescence is a widely noted structural alteration. Formation of gel phase and accumulation of gel-phase forming lipids were initially demonstrated in senescing bean cotyledons (McKersie et al., 1978; Pauls and Thompson, 1984) by wide-angle X-ray diffraction and electron paramagnetic resonance techniques. These studies were conducted using isolated microsomes or with reconstituted lipids after their isolation from the membrane. Freeze fracture studies on carnation petals also revealed the formation of gel phase during flower development (Paliyath and Thompson, 1990). During such studies, petal fragments were frozen in liquid propane and fractured in a specialized apparatus maintained at −180°C, by circulating liquid nitrogen. With this procedure, the fracture plane passes through regions with the weakest structural interactions, which most often occur in between the membrane bilayers. Thus, the fracture exposes the surface of the interior of the bilayer. A replica of the fracture face is made using evaporated gold and carbon, and the replica is cleaned and observed under an electron microscope to reveal surface property details. A freeze fracture image of a partially open carnation flower petal is shown in Fig. 9.2. The fracture plane reveals cellulose microfibrils of the cell wall (CW), the plasma membrane (PM), and the

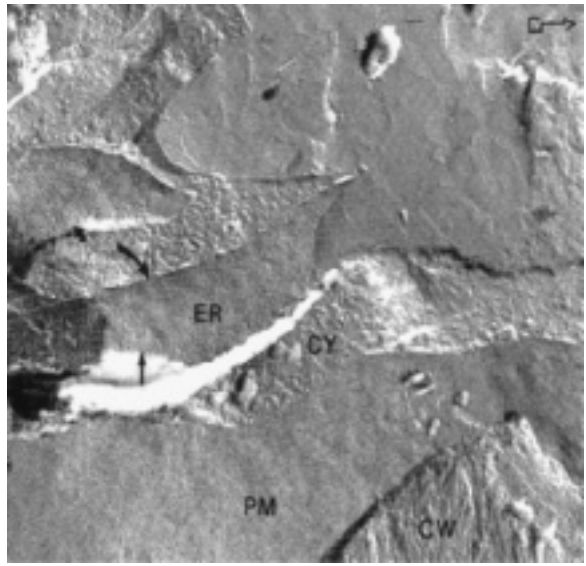


Fig. 9.2 Freeze fracture replica of a young unopened carnation flower petal. The fracture plane exposes the cell wall (CW), cytoplasm (CY), protoplasmic fracture face of the plasma membrane (PM), and the exoplasmic fracture face of the endoplasmic reticulum (ER). Arrows indicate the intramembranous particle-free regions (gel phase) on the ER. Bar = 100 nm. (Reproduced with permission from Paliyath and Thompson, 1990.)

endoplasmic reticulum (ER). The plasma membrane appears turgid and contains numerous proteins, indicative of an ideal membrane. The endoplasmic reticulum, however, includes vacant areas indicative of gel-phase lipid. During gel-phase formation, the mobility of the fatty acyl chains is reduced, making them more rigid (less fluid). Proteins that are excluded from the gel-phase accumulate in the surrounding liquid crystalline phase. The interface between the gel phase and the liquid crystalline phase does not pack very well, and this causes leakiness in the membrane. It is interesting to note that the very first symptoms of gel-phase formation are noticed on the endoplasmic reticulum and not on the plasma membrane. However, as the flower develops further (fully open), even the plasma membrane shows gel-phase areas (Fig. 9.3). The surface of the plasma membrane is frequently distributed with pitlike structures, which may represent areas containing damaged lipids in the form of microvesicles that are excluded from the membrane (Yao et al., 1991). Large vesicular structures (V) are also visible lying close to the plasma membrane.

The degradation of membrane lipids is an essential feature of senescence and signal transduction pathways that occur in response to hormones and environmental stress (Paliyath and Droillard, 1992; Chapman, 1998; Wang, 2002; Bargmann and Munnik, 2006; Wang et al., 2006). After evaluating the pattern of lipid catabolism in microsomes from various tissues, a pathway for the catabolism of phospholipids was developed in senescing systems that involves the sequential action of enzymes such as phospholipase D (PLD), phosphatidate phosphatase, lipolytic acyl hydrolase (LAH), and lipoxygenase (Fig. 9.4). PLD is the key enzyme of the pathway since none of the following enzymes can directly act on phospholipids, though there are exceptions such as the potato and carnation LAHs (Galliard, 1980; Hong et al., 2000). PLD is also stimulated by physiologically elevated levels of calcium and low cytosolic pH. Such conditions can occur during senescence when membrane

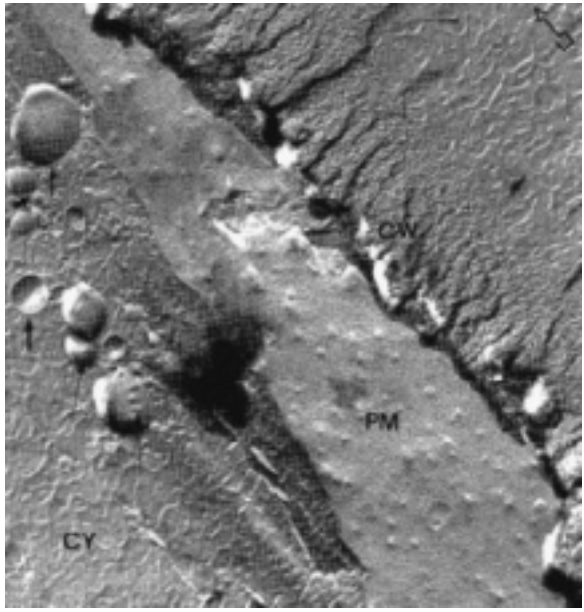


Fig. 9.3 Fracture face of a fully open carnation flower petal showing cell wall (CW), protoplasmic fracture face of the plasma membrane (PM), cytoplasm (CY), and vesicles in the cytoplasm (thick arrows). Bar = 100 nm. (Reproduced with permission from Paliyath and Thompson, 1990.)

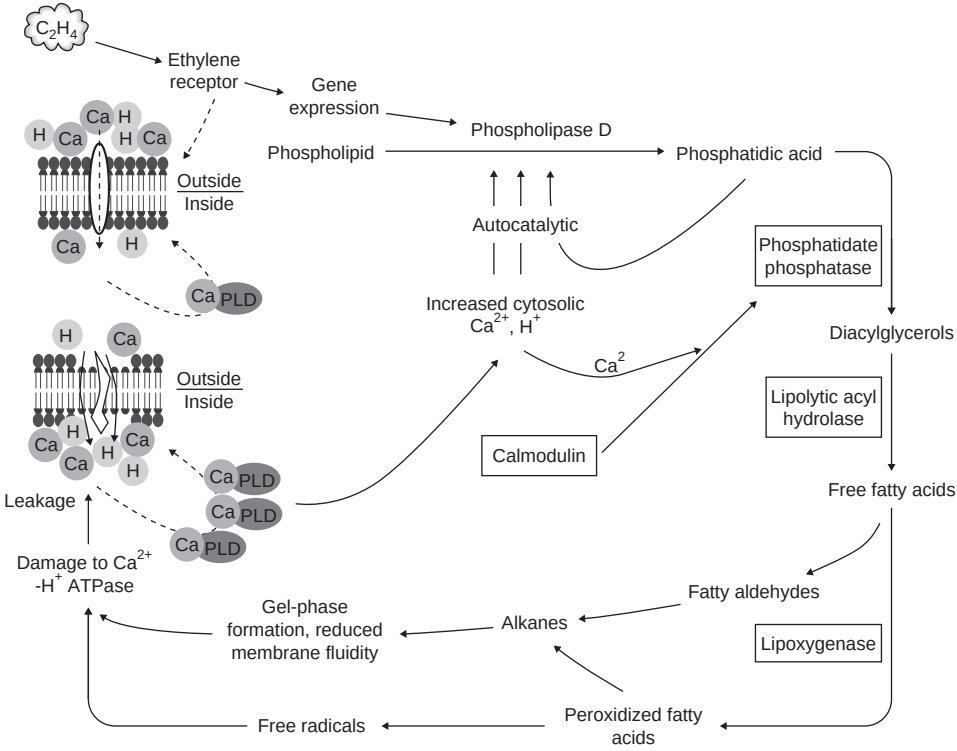


Fig. 9.4 Schematic representation of various reactions involved in membrane deterioration. The autocatalytic nature of the cycle derives from the accumulation of lipid degradation products in the membrane that causes progressively increasing membrane destabilization and loss of membrane compartmentalization. Part of this cycle is also shared with phospholipid biosynthetic pathways.

compartmentalization is disrupted, resulting in leakage of calcium ions and hydrogen ions from their storage compartments such as the cell wall and the vacuole. In addition, the activities of key plasma membrane ATPases, such as the calcium and proton ATPase that extrude the ions from the cytosol to the cell wall space, are negatively affected during senescence or after ethylene treatment as demonstrated in carnation flower petals (Paliyath and Thompson, 1988; Paliyath et al., 1997). Thus, reduced ATPase activity can also result in a buildup of calcium ions and hydrogen ions in the cytoplasm. Such conditions lead to the autocatalytic progression of membrane lipid degradation once it has been initiated by hormones (ethylene and abscisic acid) or stress. Although there is a clear link between the promotion of senescence by ethylene and enhanced membrane deterioration, the complete sequence of signal transduction events involved in this link has not yet been established.

9.3.1 Changes in PLD activity during ripening

Previous studies have attempted to correlate increased membrane deterioration that occurs during ripening and senescence to increased phospholipase D activity. Although such an increase in PLD activity was noticeable in some senescing systems such as broccoli florets (Deschene et al., 1991), it was not as distinct in systems such as carnation flower petals (Paliyath et al., 1987) and tomato fruit (Jandus et al., 1997). Thus, increased phospholipid degradation that occurs during ripening/senescence was linked to the activation of PLD by factors such as increase in cytosolic calcium and a decrease in pH, membrane rigidification, and fatty acid retailoring that increases the availability of preferred PLD substrates (Brown et al., 1990). We have examined PLD activity during fruit development using cherry tomatoes, where the developmental stages are physiologically more precise and distinguishable. PLD activity was determined in subcellular fractions comprising mitochondrial membranes, microsomal membranes, and the cytosol (Fig. 9.5). Mitochondrial PLD activity remained

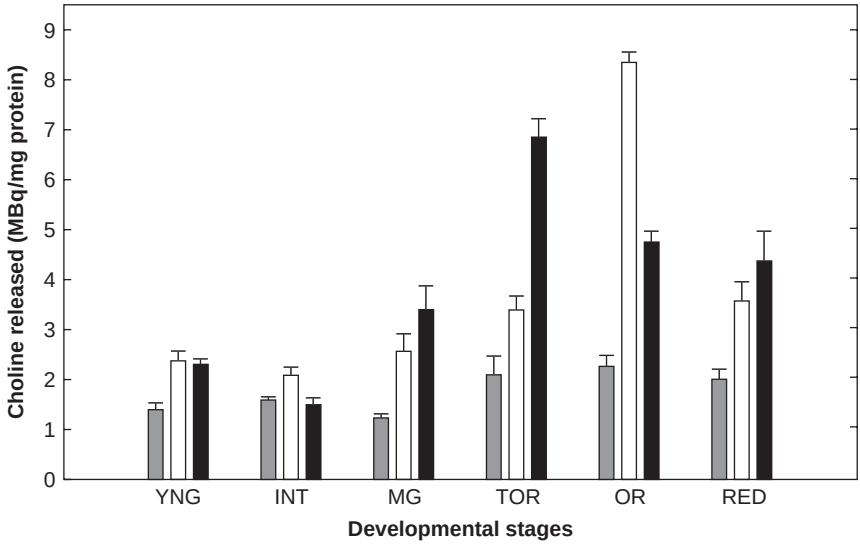


Fig. 9.5 Changes in PLD activity during development of cherry tomato. PLD activity in mitochondrial (grey), microsomal (unshaded), and cytosolic (dark) fractions was measured at young (YNG), intermediate (INT), mature green (MG), turning orange (TOR), orange (OR), and red (RED) stages. (Reproduced with permission from Pinhero et al., 2003.)

nearly constant throughout the developmental stages studied. Cytosolic PLD activity started to increase at the mature green stage, peaked at the turning orange stage, and started to decline at the orange and red stages. PLD activity associated with microsomal membranes, which remained nearly unchanged until the mature green stage, started to increase at the turning orange stage and peaked at the orange stage, temporally coinciding with the decline in cytosolic PLD. Thus, increased membrane association appears to be a major mode of developmental regulation of PLD activity in tomato fruit.

By contrast to tomato, which is a climacteric fruit, strawberry fruits are nonclimacteric and largely unresponsive to ethylene.

Fruit softening in strawberries has been primarily coined to the activities of polygalacturonases that result in cell separation, although recent reports have shown that β-xylosidase (Bustamante et al., 2006) and expansins (Dotto et al., 2006) could also be important in cell wall modification. To evaluate the potential role of PLD activity in fruit development and ripening, PLD activity was analyzed at various stages of development in two strawberry cultivars, Aromas and Seascape (Fig. 9.6). PLD activity was analyzed by monitoring the liberation of radiolabeled choline from dipalmitoyl phosphatidylcholine provided as an external substrate. The strawberry fruits were homogenized and subjected to differential centrifugation to separate mitochondrial membrane, microsomal membrane, and soluble fractions. The soluble fraction from strawberry fruit contained very little protein and PLD activity and was not used for further analyses. By contrast, the mitochondrial fraction (15,000 $\times$ g) and the microsomal fraction (105,000 $\times$ g) consistently showed detectable levels of activity. Various stages of strawberry fruit development are designated as 1—young immature (G1); 2—young expanding (G2); 3—mature white (W); 4—turning orange stage (T); and 5—firm ripened (R) stages. In general, PLD activity was higher in "Seascape" at all stages except the mature white stage (W). There was very little change in mitochondrial PLD activity during development of "Aromas" fruit. The microsomal PLD activity increased during fruit development and reached a maximum level of 28 nmol choline released per mg protein in 30 min at the mature white stage (W). PLD activity declined further during fruit development and ripening. PLD-specific activity in the microsomal fraction was the highest at the young immature stage (G1) in "Seascape," after which it declined to nearly half of its original specific activity. Mitochondrial PLD activity also showed a decline from G1 to G2; however, it continued to increase during further development and reached a maximal value of nearly 29 nmol choline released per mg protein at the turning orange stage (T; Fig. 9.6b). These results suggested that PLD activity increased during strawberry fruit development and ripening and thus may play a key role in fruit softening, as in tomato.

9.3.2 Properties of strawberry PLD

Properties of strawberry PLD were analyzed using mitochondrial and microsomal membranes isolated from the fruit. The release of radiolabeled choline from dipalmitoyl phosphatidylcholine (DPPC) in the presence of strawberry microsomal and mitochondrial membrane under various conditions was quantified.

9.3.2.1 Regulation of PLD by pH

Several previous studies have indicated that PLD is stimulated under acidic conditions (Galliard, 1980), and in particular, the relatively abundant alpha-type PLDs are activated by

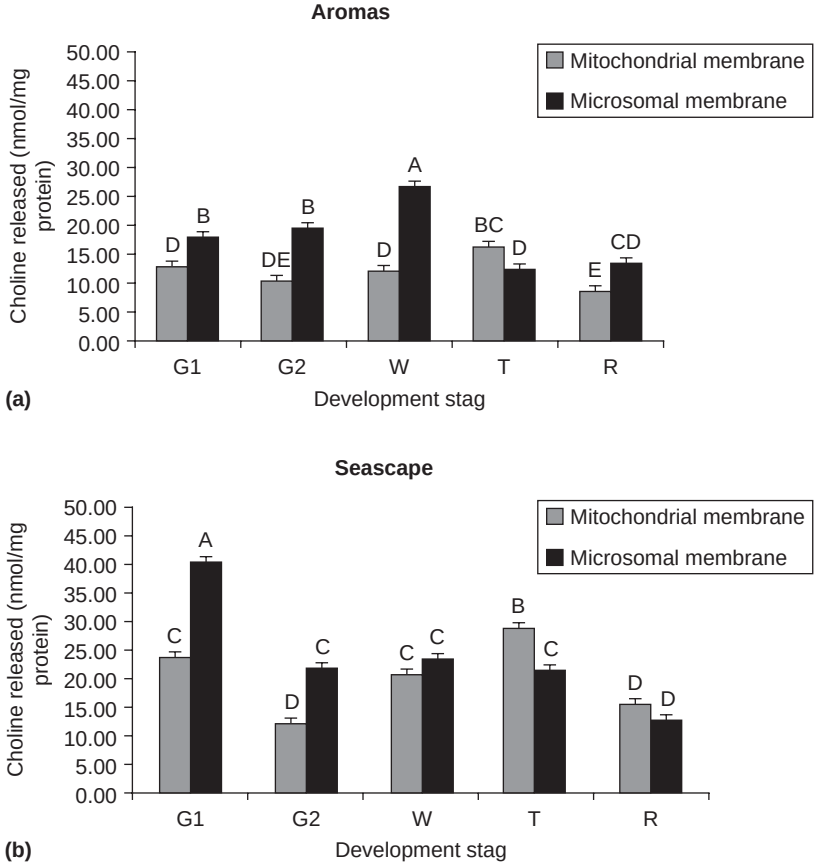


Fig. 9.6 Stage-specific changes observed in PLD activity of strawberry varieties (*Fragaria ananassa* Duch. cv. "Aromas" and "Seascape"). Fruits were collected at various developmental stages for the isolation of mitochondrial and microsomal membranes, designated as 1—young immature (G1); 2—young expanding (G2); 3—mature white (W); 4—turning orange stage (T); and 5—firm ripened stage (R). PLD activity was measured by the release of radiolabeled choline from 16:0/16:0 phosphatidylcholine (L_3-phosphatidyl (*N*-methyl-^{3}H) choline, 1,2-dipalmitoyl) in a 1-mL reaction mixture. The values are mean ±SE from three separate experiments. The histograms with different alphabets indicate that the values are significantly different at $p \leq 0.05$ (generalized linear model procedure, SAS programme, SAS Institute Inc.). (Reproduced with permission from Yuan et al., 2005.)

micromolar levels of calcium plus PIP_2 at a pH of 4.5–5.5 (Wang, 2000). The physiological implication of this effect is that PLD action will be stimulated under conditions when membrane compartmentalization is lost or the cytosol is acidified as happens during ripening and senescence. In vivo, PLD is compartmentalized in tomato fruit, and can be visualized by immunochemical localization in the cytosol, plasma membrane, endoplasmic reticulum, vacuole, and mitochondria (Pinhero et al., 2003). As well, during fruit development there is increased binding of PLD to the ER and plasma membrane, potentially in response to increased cytosolic calcium (Pinhero et al., 2003; Yuan et al., 2006a). Thus, under in vivo conditions, PLD is not exposed to acidic conditions except when it is compartmentalized in the vacuole or when it is exported into the cell wall compartment (Yuan et al., 2006a).

Strawberry is a very acidic fruit, and the organic acids that cause this acidity are sequestered in the vacuole. Thus, it was of interest to study the pH responses of PLD in strawberry fruit (Yuan et al., 2005). The mitochondrial PLD was optimally activated at pH values of 5.5 and 6.5. The microsomal PLD also showed dual optimal values of activation at pH values of 5 and 7, respectively. Similar pH activation profiles have been observed in tomato microsomal membrane (K. Tiwari, unpublished).

9.3.2.2 Kinetic analysis of PLD

PLDs belong to the class of phosphohydrolases with a broad substrate specificity, and can catalyze hydrolysis of head groups from phospholipids such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylglycerol. Under nonstimulated physiological conditions, a considerable proportion of PLD may occur in the cytosol. PLD is ineffective in the cytoplasm and does not exert its activity unless bound to the membrane. Moreover, in a membrane-bound state, PLD is likely to encounter a variety of phospholipids, toward which, it may show substrate preferences based on head group and unsaturation of acyl chains (Brown et al., 1990; Paliyath et al., 1995; Pappan et al., 1998). Thus, in the true sense, absolute kinetic parameters of PLD cannot be deciphered unless the PLD molecule becomes membrane-localized as observed under in vivo conditions. Nevertheless, analysis of substrate–velocity relationships under in vitro conditions may provide insights into the kinetic properties of PLD localized in different compartments. To study these aspects, mitochondrial and microsomal membranes were incubated with varying concentrations of DPPC prepared from a mixture of unlabeled and radiolabeled DPPC (1 nmol PC/3.7 kBq). Enzyme activities of both mitochondrial and microsomal PLD followed Michaelis–Menten kinetics. PLD activity in the microsomal fraction showed a linear increase with increasing DPPC concentration ranging from 0 to 200 μmol attaining a maximal V_{max} value of >300 nmol/mg protein per 15 min. When DPPC concentration was increased above 200 μM, PLD activity decreased (Fig. 9.7, top panel). By contrast, PLD activity in the mitochondrial fraction was considerably lower, showed only a minor increase in activity with increasing substrate concentration (Fig. 9.7, top panel), and reached a maximal velocity of 50 nmol/mg protein per 15 min between 200 and 250 μM substrate concentration, sixfold lower than the maximal activity exhibited by microsomal PLD. These results suggested that inherent differences might exist in the kinetic properties of mitochondrial and microsomal PLD.

Transformation of the substrate–velocity data through a Lineweaver–Burke plot provided further understanding of the kinetic properties of PLD (Fig. 9.7, bottom panel). Analysis of the $1/v$ versus 1/[PC] plots was typically linear; the $1/v$ intercepts ($1/V_{max}$) and the X-axis intercept (1/[PC] or $[-1/K_m]$, -0.00878 for mitochondrial PLD, -0.00361 for microsomal PLD) showing differences in kinetic properties between mitochondrial and microsomal PLD. The kinetic parameters obtained from the Lineweaver–Burke plots are given in Table 9.1. The V_{max} of microsomal PLD (44.44 nmol/mg protein per minute) was about 12-fold higher than that of the mitochondrial PLD (3.75 nmol/mg protein per minute). K_m values for the mitochondrial PLD and microsomal PLD were 114 and 277 μM, respectively. The specific constant (V_{max}/K_m) of microsomal PLD was fivefold higher than that of mitochondrial PLD, suggesting that PLD in microsomal membranes has a higher catalytic activity than that of PLD in mitochondria.

Table 9.1 Kinetic parameters of PLD toward dipalmitoylphosphatidylcholine

	Mitochondrial PLD	Microsomal PLD
V_{max} (nmol/mg protein min)	3.75	44.44
K_m (μM)	114	277
V_{max}/K_m	0.033	0.160

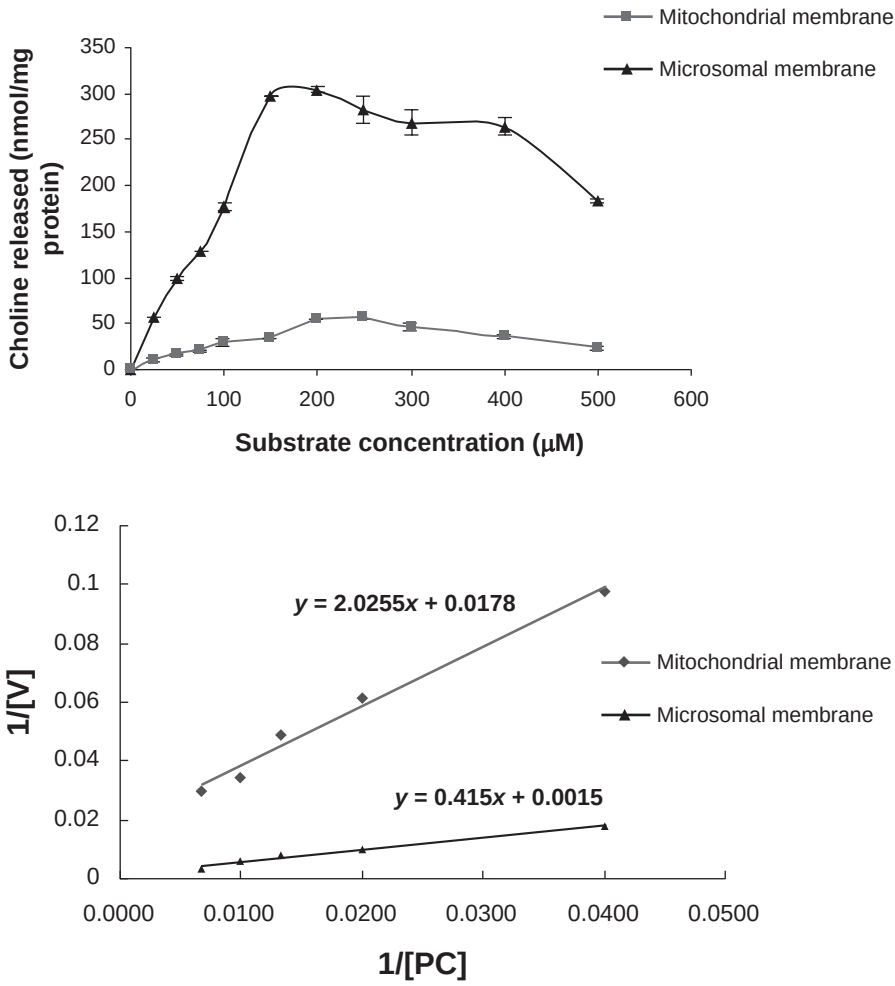


Fig. 9.7 Substrate–velocity plot (top panel) of PLD activity in mitochondrial and microsomal membrane fractions of strawberry fruit. Phospholipase D activity was measured by the release of radiolabeled choline from 16:0/16:0 phosphatidylcholine (L_3-phosphatidyl (*N*-methyl-^{3}H) choline, 1,2-dipalmitoyl) in a 1-mL reaction mixture resulting from a 15-min reaction period. The substrate was prepared by mixing cold PC and radiolabeled PC in a ratio of 1 nmol/3.7 kBq, also containing 0.1% Triton x-100 and 50-mM Hepes, pH 7.0 (final). Appropriate volumes of the substrate were added to the reaction mixtures to give the desired concentrations. A separate blank without the enzyme was made for each concentration of the substrate used to correct for the enzyme activity. The values are mean ±SE from three separate experiments. Lineweaver–Burk plot of the data is given in the bottom panel without taking the plateau region. (Reproduced with permission from Yuan et al., 2005.)

9.3.2.3 Activation of PLD by calcium

Stimulation of PLD by calcium may arise through more than one mechanism. Two possibilities are calcium-dependent membrane association, which increases enzyme substrate interactions and substrate availability, and direct activation of the enzyme by calcium. PLD activity in strawberry fruit membranes was also stimulated by calcium (Fig. 9.8, top panel). Both microsomal and mitochondrial PLD were maximally stimulated (sixfold stimulation for microsomal membranes, threefold stimulation for mitochondrial membranes) between 1 and 3-mM added calcium. The assay mixture contained 0.2-mM ethylene glycol-bis-β-aminoethyl ether, N,N,N',N' tetra-acetic acid (EGTA) that acts as a calcium-buffering agent and provides precise levels of free calcium, especially at low levels of added calcium. Microsomal membranes showed nearly fourfold stimulation between 0.15- and 0.25-mM added

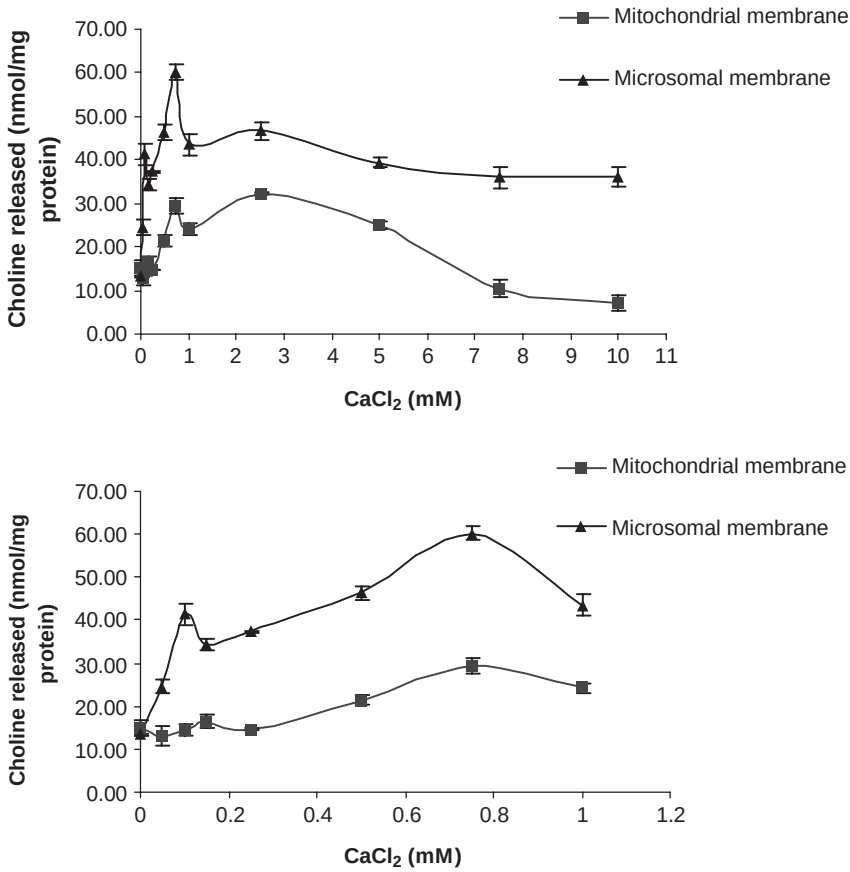


Fig. 9.8 Effect of increasing calcium on PLD activity in mitochondrial and microsomal membrane of strawberry fruit. Precise levels of free calcium levels were obtained by including 0.2-mM EGTA in the assay mixture. Under these conditions, free calcium concentrations for 100, 150, 200, and 250 μM added calcium ranged from $\leq$1, 1, 15, and 40 μM, respectively. PLD activity was measured by the release of radiolabeled choline from 16:0/16:0 phosphatidylcholine (L_3-phosphatidyl (N-methyl-^{3}H) choline, 1,2-dipalmitoyl) in a 1-mL reaction mixture resulting from a 15-min reaction period. The lower panel is an expansion of lower concentrations of calcium shown in the top panel. The values are mean $\pm$SE from three separate experiments. (Reproduced with permission from Yuan et al., 2005.)

calcium (Fig. 9.8, bottom panel), which provides free calcium in the range of $\leq$1 to 40 μM. Nearly threefold stimulation in PLD activity is observed at free calcium in the range of 1 μM (0.2-mM added calcium and 0.2-mM EGTA), which is in the range of physiologically activated levels of calcium (Felle, 1988; Yuan et al., 2006b). Thus, microsomal PLD is stimulated at low micromolar levels of calcium, implying a potential role in signal transduction processes. By contrast, mitochondrial PLD activity did not show any significant promotion at these levels of calcium (Fig. 9.8). Maximal stimulation of mitochondrial PLD was observed between 1 and 3 mM free calcium.

9.4 Subcellular localization of phospholipase D

PLD is a soluble enzyme. However, analysis of PLD activity in subcellular fractions revealed the presence of PLD activity in mitochondrial, endoplasmic reticulum, and plasma membrane fractions. Membrane-associated PLD activity increases during ripening and after wounding. PLD was localized by immunoelectron microscopy in germinating castor bean tissue, and was shown to be associated with the plasma membrane and the vacuole (Xu et al., 1996). After wounding of castor bean leaves, lipolysis by PLD increased sharply following calcium-mediated translocation from the cytoplasm to microsomal membranes (Ryu and Wang, 1996). In rice, PLD was localized in cell walls, membranes, and chloroplasts (McGee et al., 2003), and was recruited to the plasma membrane at the point of adhesion of a bacterial pathogen (Young et al., 1996). In tomato fruits, PLD was localized in cytoplasm, plasma membrane, endoplasmic reticulum, mitochondria, and nuclear membrane (Pinhero et al., 2003). During early stages of development, PLD was primarily localized in the cytoplasm. As development progressed, a relatively large number of PLD polypeptides were observed in the endoplasmic reticulum (Fig. 9.9). In ripened strawberry fruit, a large number of PLD molecules were localized in the cell wall space (Yuan et al., 2006a). This may represent an advanced stage where cell permeability is compromised and the PLD molecules have leaked out of the cell or possibly were transported by exocytosis.

9.5 Characteristics of phospholipase D

PLD (EC 3.1.4.4) is a key enzyme that catalyzes the hydrolysis of membrane phospholipids yielding PA and a hydrophilic head group (Galliard, 1980). PLD in plants was originally proposed to be important in phospholipid catabolism, initiating a lipolytic cascade in membrane deterioration during senescence and stress (Paliyath and Droillard, 1992). PLD could also be involved in phospholipid turnover that maintains cell viability and homeostasis (Dawidowicz, 1987). Recent studies in plants indicate that PLD action plays an important role in transmembrane signaling and cellular regulation (Wang, 2002, 2005). Activation of PLD generates lipid messengers, most importantly PA, which mediate an array of physiological responses (Wang, 2005; Bargmann and Munnik, 2006; Wang et al., 2006).

The role of phospholipase D in the initiation of membrane deterioration during ripening and senescence has been well recognized (Paliyath and Droillard, 1992). About 15–25% loss of total phospholipids occurs during full ripening of tomato fruit pericarp tissue, with a coincident increase in PA, suggesting that PLD is involved in membrane degradation (Güçlü et al., 1989; Whitaker, 1994). Moreover, all the enzyme activities involved in the senescence cascade, including PLD, have been demonstrated in tomato fruit microsomal

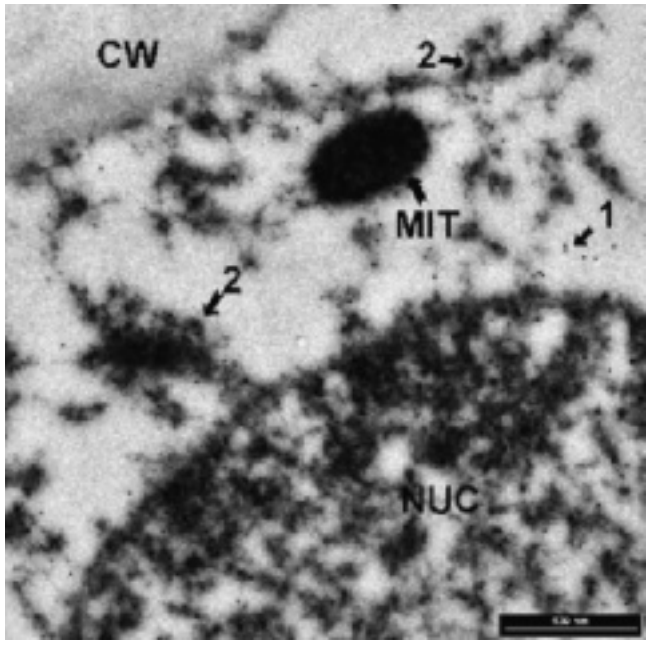


Fig. 9.9 Immunohistochemical localization of phospholipase D in tomato fruits. The sections were incubated with polyclonal antibodies raised against rapeseed phospholipase D and localized with goat antirabbit 1 gG coupled to 10-nm gold particles. The gold particles can be visualized in the cytosol (1), bound to the membrane (2), attached to the nuclear membranes (Nuc), and mitochondria (Mit). CW, cell wall. (Reproduced with permission from Pinhero et al., 2003.)

membranes (Todd et al., 1990, 1992; Pinhero et al., 2003). Since the activity of PLD appears to be the key step regulating the sequence of enzyme reactions and the flow of metabolites through the catabolic pathway (Fig. 9.4), this indirectly implies that controlling the activity of PLD is critical for enhancing the shelf life and quality of fruits and vegetables. Therefore, attempts have been made to clone *PLD* genes from fruits and vegetables and to understand their regulation during ripening and senescence. PLD activity in membrane preparations of germinating bean cotyledons, tomato fruit, strawberry fruit, and carnation flower petals was stimulated at physiologically elevated micromolar levels of calcium, which suggested that phospholipase D activation may be mediated through calcium signal transduction events. This proposed mechanism is currently being studied.

Studies on the properties and regulation of phospholipase D date back several decades, and the special enzymological property of transphosphatidylation was well recognized (Galliard, 1980). Recent advances on the physiological and molecular aspects of PLD have been summarized by Wang (1999, 2000, 2001, 2002, 2005). The functional significance of different forms of plant PLDs, in relation to developmental processes and signal transduction, appear to result from their differential ability to bind to the membrane, substrate specificity, and activation by calcium, low pH, phosphatidylinositol-4,5-bisphosphate (PIP_2), and several other factors. Complimentary DNA encoding several PLDs have been cloned, sequenced, expressed, and their physiological roles studied. These include PLDs from plants such as *Arabidopsis*, rice, maize, cabbage, castor bean, tomato, strawberry,

melon, and others. In recent years, multiple functional roles for PLD in plants have been demonstrated, including cell signaling in response to molecules such as abscisic acid (ABA) and fungal elicitors, programmed cell death, root hair development, and stress responses such as development of freezing or drought tolerance. Various plant PLDs have highly conserved regions that contribute to their enzyme function, as well as regions that differ from one another that provide the diverse functional characteristics in response to cellular and environmental signals. Plants possess a highly diverse *PLD* gene family. *Arabidopsis*, which has served as a model plant system, contains 12 *PLD* genes. By contrast, there are 2 *PLD* genes in mammalian systems and 1 in yeast. The 12 *PLD* genes from *Arabidopsis* are grouped into the alpha (3), beta (2), gamma (3), delta (1), epsilon (1), and zeta (2) types. Of these, only the two zeta-type PLDs contain plextrin homology (PH) plus phox homology (PX) domains in the N-terminal region, which are conserved in PLDs from other kingdoms. The remaining 10 *Arabidopsis* PLDs contain a C2 domain at the N-terminus, which is a calcium-dependent phospholipid-binding structural fold present in a number of lipid signaling and metabolic proteins, but unique to plant PLDs (Wang et al., 2006). These differences, along with differences in internal motifs, create the diversity in plant PLD structure and function. In addition to the conserved pair of PLD active site (HxKxxxxD) domains, different PLDs possess motifs that interact with calcium, polyphosphoinositides, G-proteins, and other factors. Phospholipases are soluble proteins, and their translocation to the membrane on physiological stimulation may be achieved through conformational changes in the C2 domain on binding to calcium released into the cytosol, or by other changes such as the biosynthesis of phosphorylated inositol phospholipids on the membrane that serve as binding sites for the PH/PX super-fold in zeta-type PLDs. In addition, the concentration of calcium ions required for activation varies widely with the type of plant PLD, due at least in part to modifications in amino acid sequences in the C2 domain, and there can also be a marked influence of pH on activity and calcium binding.

9.6 Cloning and homology of tomato, strawberry, and melon *PLD* alpha cDNAs

Following cloning of the first phospholipase D (*PLD*) gene from castor bean (Wang et al., 1994), there has been substantial progress in determining physiological roles of members of the plant *PLD* gene family, now known to comprise six classes: alpha, beta, gamma, delta, epsilon, and zeta (Bargmann and Munnik, 2006; Wang et al., 2006). Most notably, phosphatidic acid derived from PLD hydrolysis of PC and other phospholipids is an important signaling molecule that mediates responses to various types of biotic and abiotic stress (Wang, 2002, 2005; Bargmann and Munnik, 2006; Wang et al., 2006). Despite this progress, however, one of the earliest roles ascribed to PLD, that is, initiator of the cascade of phospholipid catabolism in senescing plant tissues (Paliyath and Droillard, 1992), has received relatively little attention. PLDs of the alpha class, typically the most abundantly expressed and accounting for most of the total activity (Fan et al., 1999), are the best candidates to perform this function. Although antisense knockout of *AtPLDα1* did not alter natural senescence, however, it did delay ethylene- and abscisic acid–induced senescence of detached leaves of *Arabidopsis* (Fan et al., 1997). This finding indicates a likely role of PLD in postharvest senescence of fresh fruits and vegetables, particularly in climacteric fruits such as tomato, which produce high levels of ethylene. PLD activity estimated in

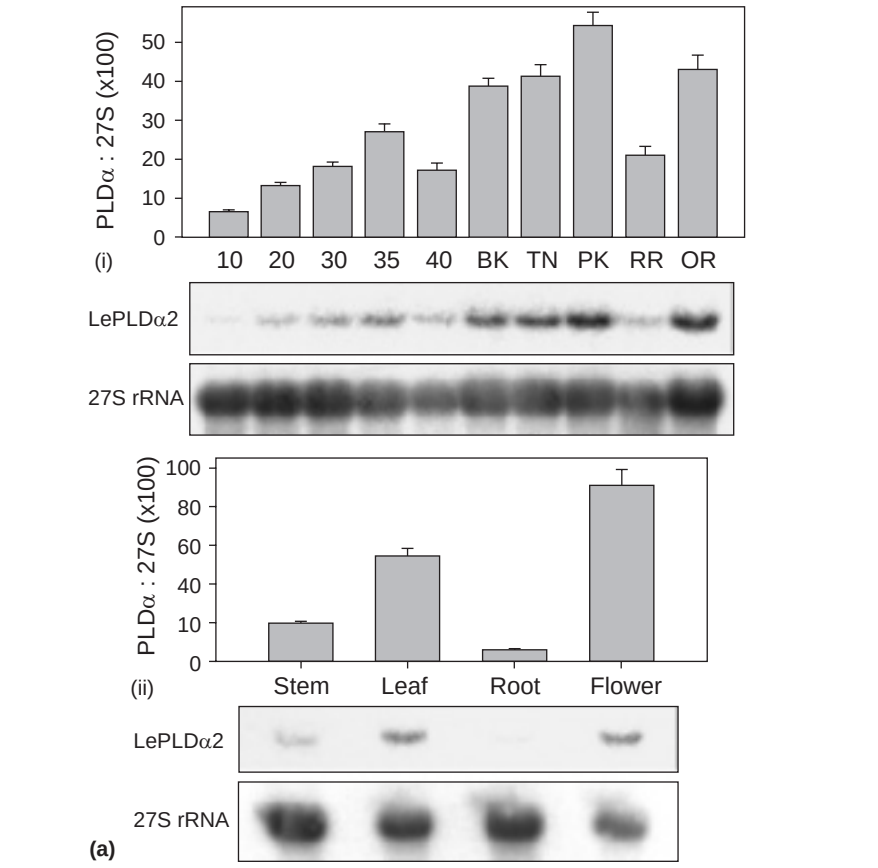
strawberry fruit membranes may also be due to PLD alpha. Western blot analysis of strawberry membrane proteins using polyclonal antibodies raised against rapeseed PLD alpha (Novotna et al., 1999) showed a predominant cross reactivity with a 92-kDa protein (Yuan et al., 2005), and nothing above this molecular mass, suggesting that PLD beta (112 kDa) and gamma (98 kDa) isoforms may occur below detectable levels, or do not react with the PLD alpha antibodies. As well, the PLD alpha 1 encoded by *FaPLDα1* from strawberry fruit (Yuan et al., 2005) is very similar to that encoded by *LePLDα1* from tomato. In general, strawberry PLD alpha is also similar to tomato PLD alpha in its properties and regulation by calcium (Tiwari et al., unpublished). Despite differences in kinetic properties, strawberry fruit mitochondrial PLD appears to be similar to microsomal PLD in its molecular mass, about 92 kDa, which is indicative of an alpha-type PLD (Yuan et al., 2006a). The different kinetic properties of mitochondrial versus microsomal PLD may arise from posttranslational modifications, or the presence/absence of particular modulatory lipids or other effectors. Irrespective of whether a fruit is climacteric (tomato), weakly climacteric (hybrid honeydew melon), or nonclimacteric (strawberry), PLD may play a key role in the initiation and progression of ripening and senescence.

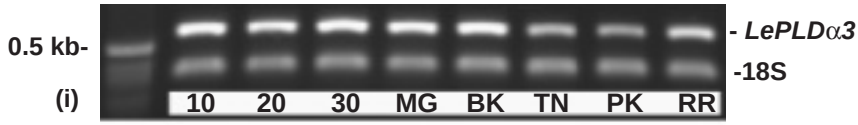
Three isogenes encoding PLD alphas (referred to here as *LePLDα1*, *LePLDα2*, and *LePLDα3*) have been cloned from tomato and further studied (Table 9.2). *LePLDα1* (Almquist and Paliyath, 2000; GenBank accession number AF201661) was cloned using a novel strategy for the design of primers known as the CODEHOP program (Rose et al., 1998), by which PCR primers were designed based on highly conserved regions of *Arabidopsis thaliana* PLD alpha-, beta-, and gamma-encoded amino acid sequences aligned using the Clustal W program (Altschul et al., 1997). A CODEHOP primer is degenerated at the 3′ core region, with a length of 11–12 bp spanning four codons representing highly conserved amino acids, and nondegenerate at the 5′ consensus clamp region, which is variable in length. The hybrid structure (5′ consensus and 3′ degenerate) of CODEHOP primers has been reported to result in specific amplification of cDNA during early cycles of PCR, and selective amplification of products during later cycles. Forward and reverse primers were generated and PCR performed using tomato root cDNA (reported in Pinhero et al., 2003). Initially this step amplified two cDNA fragments: one 883-bp long corresponding to a PLD alpha and another 936-bp long corresponding to a PLD beta or second PLD alpha. Forward

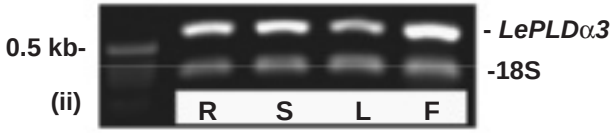
Table 9.2 Percent deduced amino acid sequence identity/similarity among seven PLD alpha full-length cDNAs from tomato (*LePLDα1*, *LePLDα2*, and *LePLDα3*), strawberry (*FaPLDα1*), honeydew melon (*CmPLDα1* and *CmPLDα2*), and cucumber (*CsPLDα1)*

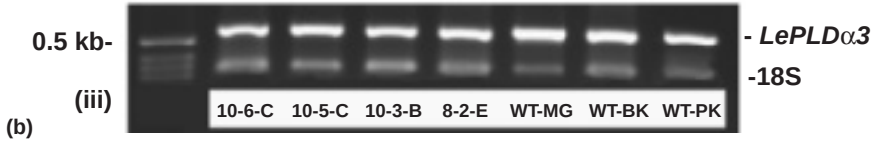
	LePLDα1	LePLDα2	LePLDα3	FaPLDα1	CmPLDα1	CmPLDα2	CsPLDα1
LePLDα1	—	76/87	74/86	81/89	82/92	75/98	81/91
LePLDα2	76/87	—	87/94	75/87	76/87	74/87	76/87
LePLDα3	74/86	87/94	—	73/86	74/86	71/86	74/86
FaPLDα1	81/89	75/87	73/86	—	82/91	76/88	82/91
CmPLDα1	82/92	76/87	74/86	82/91	—	78/90	97/98
CmPLDα2	75/88	74/87	71/86	76/88	78/90	—	77/89
CsPLDα1	81/91	76/87	74/86	82/91	97/98	77/89	—

The corresponding GenBank protein accession numbers are as follows: LePLDα1 = AAF17557, LePLDα2 = AAG48162, LePLDα3 = AAG45486, FaPLDα1 = AAW83125, CmPLDα1 = ABB82551, CmPLDα2 = ABS86615, CsPLDα1 = ABN13537.


PLDα : 27S (x100)
50
40
30
20
10
0
(i)
10 20 30 35 40 BK TN PK RR OR
LePLDα2
27S rRNA
PLDα : 27S (x100)
100
80
60
40
10
0
(ii)
Stem Leaf Root Flower
LePLDα2
27S rRNA
(a)


0.5 kb-
(i)
10 20 30 MG BK TN PK RR
- LePLDα3
-18S


0.5 kb-
(ii)
R S L F
- LePLDα3
-18S


0.5 kb-
(iii)
10-6-C 10-5-C 10-3-B 8-2-E WT-MG WT-BK WT-PK
- LePLDα3
-18S
(b)

and reverse primers were generated to conduct 3′ and 5′ rapid amplification of cDNA ends (RACE) using the 883-bp fragment, and a 2,430-bp full-length cDNA was isolated encoding a PLD alpha 809 amino acids long (*LePLDα1*).

In contrast with *LePLDα1*, the second tomato PLD alpha isogene, *LePLDα2* (AF154425), was cloned by probing a fruit cDNA library (Smith et al., 1998) with a 1.6-kb fragment of the open reading frame (ORF) from the castor bean PLD alpha cDNA, L33686 (detailed in Whitaker et al., 2001). Ten of 11 positive clones were from the same gene, and the longest of these was selected for sequencing. The complete cDNA was 2,863 nucleotides long, including a 2,424-bp ORF encoding a PLD alpha 807 amino acids in length (*LePLDα2*). The complete cDNA of a third tomato PLD alpha isogene, *LePLDα3* (AY013253), was cloned during the course of a study examining *PLD* gene expression patterns in tomato plants infected with a fungal pathogen or challenged with a fungal elicitor (Laxalt et al., 2001). A total of five tomato PLD cDNAs was isolated, including three PLD alphas and two betas, using degenerate primers and cDNA generated from root plus stem tissue RNA, or by probing a cDNA library produced using RNA from *Fusarium*-infected root plus stem tissue. Among the five PLD clones were a full-length PLD alpha cDNA, AY013252, nearly identical to *LePLDα1* (AF201661), and a partial PLD alpha cDNA, AY013254, which represents the same gene as *LePLDα2* (AF154425). Northern blot analysis of PLD alpha transcript levels in various tissues indicated that *LePLDα2* and *LePLDα3* are most abundantly expressed in flowers > fruit, and that levels of *LePLDα2* and *LePLDα3* mRNA increase through the orange stage of fruit ripening, remaining high in red ripe fruit. In contrast, *LePLDα1* transcript was most abundant in immature green fruit, and levels declined thereafter in ripening fruit. Whitaker et al. (2001) performed isogene-specific DNA and RNA gel blot analyses of *LePLDα2* in "Rutgers" tomato plants using a radiolabeled probe PCR amplified from the 3′-untranslated region (3′-UTR) of the cDNA. *LePLDα2* was shown to be a single copy gene that, as determined by Laxalt et al. (2001), is most abundantly expressed in floral and fruit pericarp tissues (Fig. 9.10). The abundance of *LePLDα2* transcript increased throughout fruit development from 10 days postanthesis, reaching a maximum at the pink/orange stage of ripening (Fig. 9.10a). A cDNA encoding *LePLDα3*

←

Fig. 9.10 (a) Abundance of *LePLDα2* transcript in "Rutgers" tomato fruit pericarp and locular tissues over the course of development and ripening (i) and in floral and vegetative tissues (ii). Total RNA was isolated from fruit at 10, 20, 30, 35, and 40 days postanthesis and at the breaker (BK), turning (TN), pink (PK), red ripe (RR), and overripe (OR) stages of ripening, as well as from stem, leaf, root, and flower tissues. About 20 μg per lane was run on 3% agarose gels and probed with a 3′-UTR fragment of the *LePLDα2* cDNA. The blots were then stripped and hybridized with a soybean 26S DNA probe to evaluate loading of total RNA (as indicated by levels of tomato 27S rRNA). Bar graph above shows the ratio of *LePLDα2* mRNA to rRNA ($\times$100) derived from densitometry scans of the autoradiograph bands. (b) Semiquantitative RT-PCR analysis of *LePLDα3* transcript levels in (i) pericarp tissue of wild-type (WT) "Rutgers" fruit during development and ripening; (ii) other WT plant organs including roots (R), stems (S), leaves (L), and flowers (F); and (iii) pericarp tissue of fruit from *LePLDα2* antisense lines (10-6-C, 10-5-C, 10-3-B, 8-2-E) harvested 40 days after pollination, as well as WT harvested at the mature green (MG), breaker (BK), and pink (PK) stages. Isogene-specific primers were used to generate the 0.6-kb fragment corresponding to nucleotides 333–908 of the *LePLDα3* coding region. The QuantumRNA quantitative RT-PCR kit (Ambion) was used to determine levels of *LePLDα3* cDNA relative to the control 18S cDNA. Ethidium bromide-stained gels of the RT-PCR products were used for quantification of the amplified *LePLDα3* and 18S cDNA fragments by densitometry. The left lane in each image shows the location of a 0.5-kb DNA marker. Developmental stages in (i) were 10, 20, and 30 days after pollination, mature green (MG), breaker (BK), turning (TN), pink (PK), and red ripe (RR). (Reproduced with permission from Whitaker et al., 2001.)

(806 amino acids) was subsequently cloned using RNA from "Rutgers" tomato fruit and gene-specific primers based on GenBank sequence AY013253 (Whitaker et al., unpublished). The nucleotide sequence of the *LePLDα3* open reading frame was identical to that of AY013253 reported by Laxalt et al. (2001). However, in contrast with their Northern blot results, semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) using a pair of isogene-specific primers indicated that *LePLDα3* transcript is most abundant in fruit pericarp tissue from early development (10 days after pollination) through the breaker stage of ripening, declining substantially thereafter (Fig. 9.10b). *LePLDα3* expression in organs other than fruit (Fig. 9.10b) was found to be similar to that determined for *LePLDα2* (Fig. 9.10a), with the greatest abundance in floral tissues.

More recently, our studies on the role of PLD in fruit ripening and senescence have included cloning of full-length PLD alpha cDNAs from cultivated strawberry (*Fragaria* × *ananassa*) and "Honey Brew" hybrid honeydew melon (*Cucumis melo* var. *inodorus*). The strawberry cDNA (AY758359), hereafter referred to as *FaPLDα1*, was cloned using cDNA reverse transcribed from fruit tissue RNA and degenerate primers based on two highly conserved regions of plant PLDs, followed by 3′ and 5′ RACE (Yuan et al., 2005). The ORF of *FaPLDα1* is 2,433 bp encoding a PLD alpha 810 amino acids in length. similar strategy and methodology were utilized to clone *CmPLDα1* (DQ267933) from honeydew melon, with the exception that leaf tissue RNA was used initially to isolate 5′- and 3′-end cDNA fragments (Whitaker and Lester, 2006). Eventually, the entire 2,427-bp ORF, encoding a PLD alpha 808 amino acids long, was amplified from cDNA reverse transcribed from fruit mesocarp tissue RNA using gene-specific primers. During cloning of *CmPLDα1*, a cDNA fragment representing a second *C. melo* PLD alpha isogene was also isolated. Quite recently the complete cDNA, *CmPLDα2* (EF543155), was cloned and reported in GenBank (Whitaker et al., unpublished). *CmPLDα2* includes a 2,424-bp ORF encoding an 807-amino acid PLD alpha. One additional, related PLD alpha cDNA worthy of mention was cloned from cucumber (*Cucumis sativus*) fruit and utilized in recent studies by Mao et al. (2007a, b) on the role of PLD and lipoxygenase (LOX) in development of chilling injury. The complete cDNA (EF363796), hereafter referred to as *CsPLDα1*, is 2,756-bp long and includes a 2,427-bp ORF encoding a PLD alpha 808 amino acids in length.

A series of BLASTP searches on the NCBI website (www.ncbi.nlm.nih.gov) was conducted to establish the encoded amino acid sequence identity and similarity among the seven PLD alpha genes described above from tomato, strawberry, honeydew melon, and cucumber. The results, presented in Table 9.3, show that the encoded proteins are all highly conserved, sharing a minimum of 71% amino acid identity and 86% similarity (LePLDα3 vs CmPLDα2). Notably, *LePLDα1* segregates from *LePLDα2* and *LePLDα3*, which are 87% identical, but LePLDα1 is more closely similar to *FaPLDα1*, *CmPLDα1*, and *CsPLDα1*. Not surprisingly, *CmPLDα1* and *CsPLDα1*, representing the same genus, are 97% identical. Overall, *CmPLDα2* was most dissimilar from the other six PLD alphas, sharing the closest identity with the honeydew melon isozyme *CmPLDα1*. Figure 9.11 presents a phylogenetic tree indicating primary structural relatedness of the seven encoded PLD alphas from tomato, strawberry, honeydew melon, and cucumber, as well as 18 additional PLD alphas from 13 other dicot species. It is interesting that similarity of PLD alpha primary structure is often in accord with taxonomic proximity, for example, LePLDα1 and tobacco PLDα1 (*Nicotiana tabacum*; CAB06620), and CmPLDα1 and CsPLDα1, yet isozymes from the same species can also differ substantially. Possibly, this is an indication of separate functions

Table 9.3 Chemical and physical parameters of PLD alpha proteins encoded by genes in tomato (LePLDα1, LePLDα2, and LePLDα3), strawberry (FaPLDα1), honeydew melon (CmPLDα1 and CmPLDα2), and cucumber (CsPLDα1)

	Amino acids	MM (kDa)	ASP + GLU	ARG + LYS	Acidic/ basic	Theoretical pI	Grand average hydropathicity
LePLDα1	809	92.2	121	89	A	5.39	−0.447
LePLDα2	807	92.0	112	86	A	5.59	−0.349
LePLDα3	806	92.7	105	94	A^-	6.29	−0.378
FaPLDα1	810	91.4	114	91	A	5.76	−0.395
CmPLDα1	808	92.0	122	92	A	5.37	−0.483
CmPLDα2	807	92.1	109	97	A^-	6.20	−0.398
CsPLDα1	808	91.8	122	92	A	5.37	−0.472

Parameters shown in columns 1–7 from left to right include total encoded amino acids, molecular mass (MM), sum of aspartic plus glutamic acid residues (ASP + GLU), sum of arginine plus lysine residues (ARG + LYS), acidic or basic polypeptide (A, acidic; A^-, weakly acidic), theoretical isoelectric point (pI), and grand average hydropathicity. PLD alpha protein accession numbers are shown in Table 9.2.

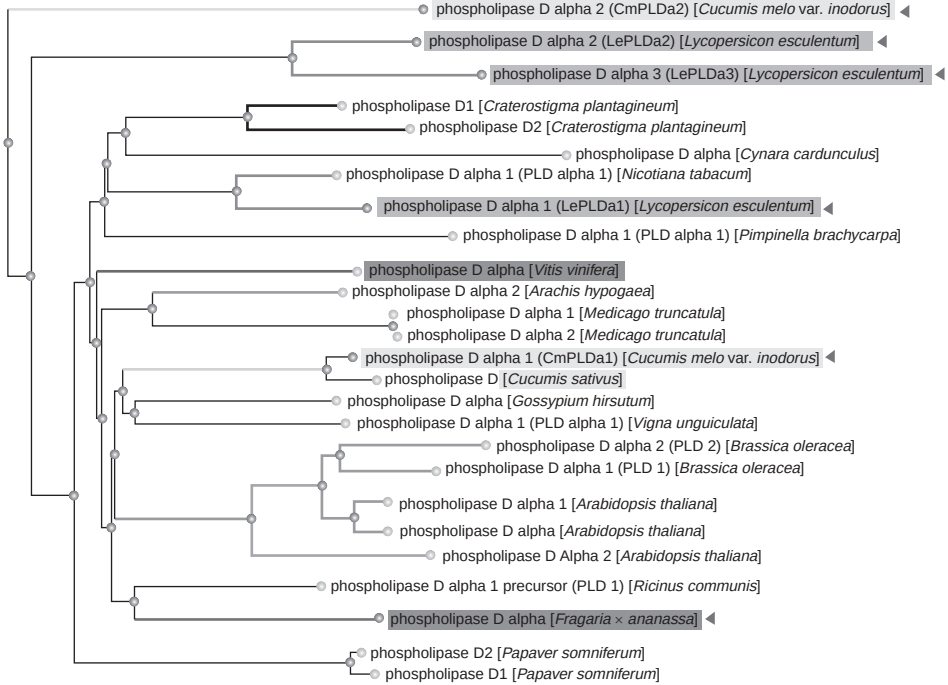


Fig. 9.11 Phylogenetic tree showing the relatedness of plant PLD alphas from 17 dicot species based on alignment of their complete deduced amino acid sequences ranging from 806 to 813 residues. Accessions with arrowheads on the right indicate the seven PLD alpha genes currently being investigated in relation to ripening and senescence of tomato (*Lycopersicon esculentum*—light gray), honeydew melon (*C. melo*—medium gray), and strawberry (*F.* × *ananassa*—dark gray) fruits. The other two highlighted accessions indicate PLD alphas cloned from tissues of grape berry (*V. vinifera*—dark gray) and cucumber fruit (*C. sativus*—light gray).

for the different isozymes in a single species. It should be noted that stacking of sequences within the phylogram does not necessarily reflect the closest structural similarity; *FaPLDα1* is most similar to *PLDα1* from castor bean (*Ricinus communis*), but is also quite similar to *CmPLDα1*, *CsPLDα1*, and *LePLDα1*. A PLD alpha from grape berry (*Vitis vinifera*; ABC59316) aligned most closely with castor bean *PLDα1*, peanut (*Arachis hypogaea*) *PLDα2* (BAE79735), and *CmPLDα1*. Aside from each other, *LePLDα2* and *LePLDα3* are most similar to peanut *PLDα2* and castor bean *PLDα1*.

9.6.1 Chemical and physical parameters of fruit PLD alphas

From the predicted amino acid sequences for PLD alphas encoded by genes in tomato (*LePLDα1*, *LePLDα2*, and *LePLDα3*), strawberry (*FaPLDα1*), honeydew melon (*CmPLDα1* and *CmPLDα2*), and cucumber (*CsPLDα1*), several structural and functional properties of these proteins were obtained using programs available on the world wide web. Table 9.3 presents a comparison of the seven PLD alphas with respect to the number of amino acids, molecular mass, sums of aspartate plus glutamate and arginine plus lysine residues, acidity, isoelectric point (pI), and grand average hydropathicity. At a first glance, the remarkable similarity of these highly conserved protein sequences is clearly evident. The total amino acids and molecular mass vary by only 0.5 and 1.4%, respectively. There are, however, some interesting differences in acidity and pI, which are determined by the relative numbers of charged acidic and basic amino acids (calculated using the Expasy ProtParam tool). LePLDα1, CmPLDα1, and CsPLDα1 are the most acidic and have the lowest, nearly identical pIs. FaPLDα1 and LePLDα2 are intermediate in acidity and pI, whereas LePLDα3 and CmPLDα2 are only weakly acidic and have the highest, closely similar pIs. In addition, although all seven PLD alphas were shown to be soluble proteins, hydropathy calculations indicated some variation in grand average hydropathicity, CmPLDα1 > CsPLDα1 > LePLDα1 having the most negative values, and LePLDα2 >LePLDα3 > FaPLDα1 = CmPLDα2 having the least negative values.

A PESTfind search (http://bioweb.pasteur.fr/seqanal/interfaces/Pestfind-simple.html) was conducted to determine potential proteolytic targeting sequences that are hydrophilic spans of 12 or more amino acids (AAs) including at least one proline, one glutamic or aspartic acid, and one serine or threonine residue, flanked by lysine, arginine, or histidine residues (Rogers et al., 1986). The search revealed one such sequence common to all seven PLDs in the range of AAs 628–649 (Fig. 9.12). The PEST score, considered as an inverse measure of the protein's half-life, was highest for LePLDα2 (17.35), lowest for LePLDα3 (6.54), and intermediate for the remaining five PLD alphas (ranging from 10.27 to 13.59). A second PEST sequence with a much lower score was detected for both FaPLDα1 (2.08) and CsPLDα1 (0.21), whereas a second sequence (AAs 605–614) with a significant score of 14.30 was identified for CmPLDα1.

9.6.2 Secondary structure, interactions, and modulation of fruit PLD alphas

The predicted nature of PLD alphas as soluble proteins with no membrane spanning domains suggests interesting secondary and tertiary structural and conformational characteristics that account for membrane association as well as binding of lipid substrates and modulators. Reverse position-specific (RPS) BLAST searches for conserved domains on the NCBI

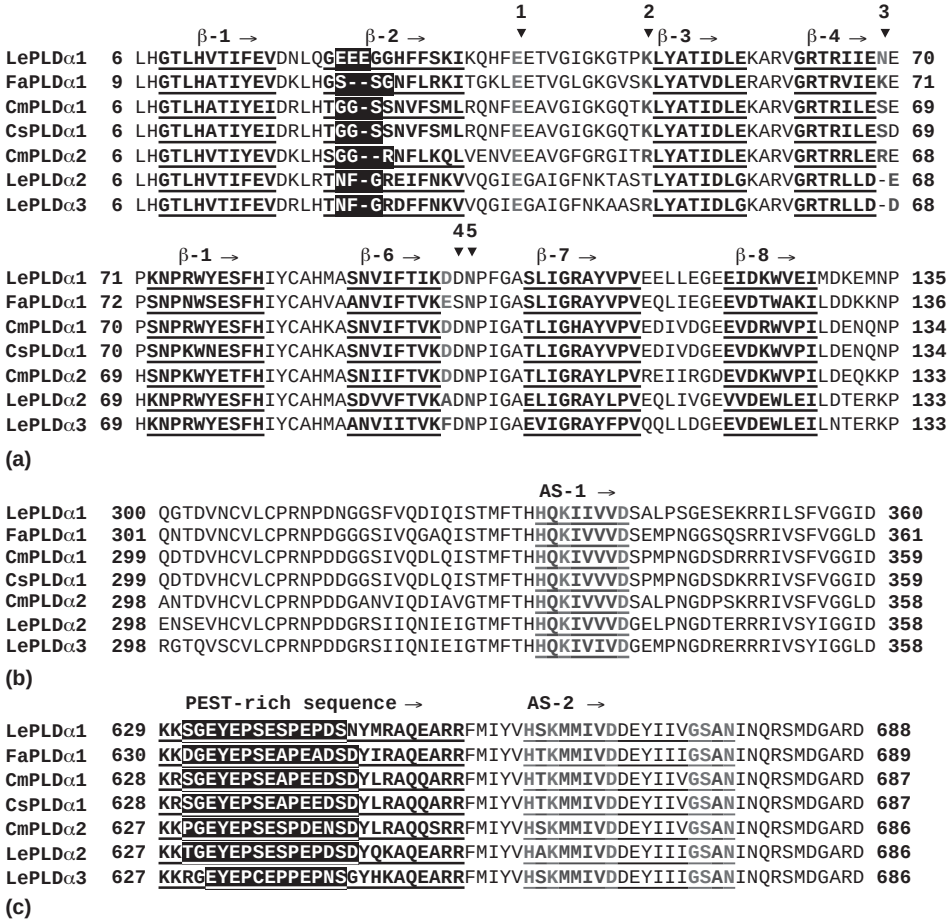


Fig. 9.12 Alignment of the encoded amino acid sequences of PLD alphas from tomato (LePLDα1, LePLDα2, and LePLDα3), strawberry (FaPLDα1), honeydew melon (CmPLDα1 and CmPLDα2), and cucumber (CsPLDα1) comparing (a) the C2 Ca^{2+}/phospholipid-binding domain, (b) the first HKD active site motif (AS-1), and (c) a PEST-rich proteolysis-targeting sequence plus the second HKD-GSN active site motif (AS-2).

website showed the presence of a C2 Ca^{2+}/phospholipids-binding domain and two PLD active site motifs, HxKxxxxD and HxKxxxxDxxxxxxGSxN (Pappan et al., 1997; Stuckey and Dixon, 1999; Wang, 2000), in each of the seven PLD alphas from tomato, strawberry, melon, and cucumber (Fig. 9.12). Despite the relatively poor conservation between PLD alphas and other plant PLDs (about 40–44% identity with PLDs beta, gamma, and delta), these features are present in all types of plant PLDs except PLD zeta, which includes plextrin and phox homology domains and lacks the C2 domain (Wang et al., 2006).

C2 domains occur in a variety of proteins that in one way or another interact with membranes. Calcium binding to specific sites in the C2 domain confers a charge to the protein, facilitating electrostatic association with anionic sites on a membrane (Ponting and Kerr, 1996; Nalefski et al., 1997). Thus, in plant PLDs including a C2 domain, calcium-activated membrane association of an otherwise soluble, cytosolic enzyme appears to involve an

electrostatic rather than a hydrophobic interaction. It was also recently shown that the C2 domain of PLD alphas possesses binding sites that enable Ca^{2+}-independent association with an aspartic protease, cardosin A, involving RGD and KGE motifs in the protease sequence (Simões et al., 2005). The C2 domain in plant PLD alphas comprises the range of amino acids from about 6 to 150 (Fig. 9.12), and as in other C2 superfamily proteins is characterized by the presence of eight beta strand motifs that form an antiparallel beta sandwich (Nalefski et al., 1997; Rizo and Südhof, 1998). The proposed locations of the eight beta strands in C2 of plant PLD alphas and betas appear to vary depending on whether modeling is based on the crystal structure of rat synaptotagmin or PLC delta 1 and $cPLA_2$ (Pappan et al., 1997; Rizo and Südhof, 1998; Zheng et al., 2000). Nevertheless, a common feature in both models is the presence of several critical amino acids that participate in binding of up to four Ca^{2+} ions and are located at specific sites on exposed loops connecting the beta sheets. In *Arabidopsis* PLD beta 1 and rat synaptotagmin, four acidic amino acid residues (glutamic acid; E or aspartic acid; D) were identified as those most involved in Ca^{2+} binding (Pappan et al., 1997). A subsequent alignment of *Arabidopsis* PLD alpha and beta with PLC delta 1 and $cPLA_2$ confirmed three of those four amino acids and identified a single asparagine (N) residue as also being important (Zheng et al., 2000). The five corresponding residues in LePLDα1 occur at AA 38, 49, 69, 97, and 99 (Fig. 9.12), and notably, acidic AAs at 49 and 99 have been substituted by basic lysine (K) and polar but uncharged asparagine (N), respectively. These, or similar substitutions seen in the seven fruit PLD alphas were thought to account for the requirement of alpha-type PLDs for millimolar rather than micromolar concentrations of calcium for activity at neutral pH (Wang, 2000, 2001; Zheng et al., 2000). However, other studies (Yuan et al., 2006a; Tiwari and Paliyath, 2007) have confirmed that strawberry PLD alpha and a tomato PLD alpha C2 domain show activation at physiologically activated, low micromolar levels of calcium suggesting the presence of high-affinity calcium-binding sites. It is interesting that the third of the four critical acidic AAs, at position 95 in LePLDα2 and LePLDα3, is substituted by nonpolar and hydrophobic alanine or phenylalanine (Fig. 9.12), raising the possibility that Ca^{2+} binding is severely compromised in these two PLD alphas. As well, LePLDα1 is the only PLD alpha of the seven to have asparagine (AA 69) at the nonacidic Ca^{2+}-binding position found in PLC delta 1 and $cPLA_2$, although all the PLD alphas have two acidic AAs (E or D) adjacent to this residue that could take part in binding calcium ions. Conceivably the C2 domains in the seven fruit PLD alphas could exist in different topologies, thus exposing different sets of calcium-binding sites, but nevertheless exhibiting conformational changes required for phospholipid (membrane) binding, some of these at physiologically activated cytosolic calcium levels.

C2 in *Arabidopsis* PLD beta was shown to have three high-affinity Ca^{2+}-binding sites with dissociation constants (K_ds) of 0.8, 2.4, and 24 μM, whereas calorimetry data indicated that C2 of *Arabidopsis* PLD alpha has 1–3 low-affinity sites with K_d in the range of 470–590 μM (Zheng et al., 2000). However, this property in *Arabidopsis* PLD alpha is a unique situation and may not be reflective of several PLD alphas that show high-affinity calcium-binding sites and are activated at micromolar calcium levels. Aside from in the C2 domain, Pappan et al. (2004) demonstrated that specific binding of calcium ions also occurs in the catalytic portion of *Arabidopsis* PLD beta 1. A bacterially expressed PLD beta 1 lacking the C2 region had much lower activity than the complete enzyme, required higher levels of calcium for maximum activity, and was activated by phosphatidylserine much more than

by other phospholipids. Presumably, Ca^{2+} binding in the catalytic domain of PLD alphas could play a part in activation, but this has not been determined experimentally.

Among the seven fruit PLD alphas aligned in Fig. 9.12, a small region of structural diversity at AAs 23–25 (26–29 in FaPLDα1) is observed at the beginning of the second beta-sheet sequence (note that this is the start of the first Ca^{2+}-binding loop in the alignment based on PLC delta 1 and $cPLA_2$ depicted in Zheng et al., 2000). Typically this region is occupied by three glycine and/or serine residues, as is the case in CmPLDα1, CsPLDα1, CmPLDα2, and FaPLDα1. LePLDα2 and LePLDα3 differ by the presence of asparagine followed by the hydrophobic amino acid phenylalanine. In marked contrast, C2 of LePLDα1 contains three acidic glutamate residues (preceding the more typical GGH). Recent investigations have shown that a tomato PLD alpha 1 C2-GFP chimeric protein expressed in *Eschericia coli* show calcium-dependent association with microsomal membrane vesicles below 1 μM level of calcium confirming the high affinity of the PLD alpha 1 C2 domains to calcium (Tiwari and Paliyath, 2007). There is some evidence that the N-terminal 30–35 AAs compose a leader sequence that is clipped from the mature enzyme in vivo (Wang, 2000), so possibly the diverse region within this leader sequence serves a targeting function, directing localization of different PLD alpha isozymes.

Another characteristic feature of PLD alphas that is likely to confer significant secondary structure is two highly conserved motifs including a relatively large number of positively charged, basic amino acids (arginine; R and lysine; K). The first motif lies within the C2 domain (Qin et al., 1997) and among the seven fruit PLD alphas (Fig. 9.12) includes 8–9 residues beginning at AA 56–59. The sequence is KARVGRTR in LePLDα1, LePLDα2, LePLDα3, CmPLDα1, and CsPLDα1, KARVGRTRR in CmPLDα2, and RARVGRTR in FaPLDα1. The second, 21-AA motif ranges from AA 239–242 to AA 259–262 and includes 3 R + 3 K in CmPLDα1 and CsPLDα1, 3 R + 4 K in LePLDα1, LePLDα2, and FaPLDα1, 3 R + 5 K in CmPLDα2, and 5 R + 3 K in LePLDα3. By virtue of their consistent presence and conserved nature, these motifs are likely to be of great significance to the conformation of PLD alpha. The abundance of positively charged residues may serve as an anchoring domain that binds to anionic lipid domains in the membrane composed of polyphosphoinositides (PIP, PIP_2, PIP_3). We propose that calcium binding to the C2 domain could expose these positively charged domains, facilitating binding to polyphosphoinositide-enriched, anionic membrane domains that are generated in response to primary stimuli. Further experimentation will be required to test this hypothesis.

Beyond the second positively charged, arginine- and lysine-rich motif, in the catalytic portion of PLD alpha, lie the two active site domains. Each has the strictly conserved HxKxxxxD motif, but the second also includes another conserved group of AAs, GSxN, such that the entire motif at the second active site is HxKxxxxDxxxxxxGSxN (Fig. 9.12). Also referred to as the phospholipase D domain (Swiss Institute of Bioinformatics), this motif is common to a broad array of phosphodiesterases that make up the PLD superfamily (Ponting and Kerr, 1996; Stuckey and Dixon, 1999; Wang, 2000). In the seven PLD alphas shown in Fig. 9.12, the first HxKxxxxD motif occurs over a range varying from AA 330–337 to AA 333–340, and among these seven sequences it differs only by the conserved substitution of one isoleucine for one valine in LePLDα1 and LePLDα3. Ten AAs downstream from this active site (AA 349–351 in LePLDα1), there is another positively charged motif consisting of KKR in LePLDα1, CsPLDα1, and CmPLDα2, RRR in CmPLDα1, LePLDα2, and LePLDα3, and SRR in FaPLDα1.

In the region between the two catalytic HKD motifs, beginning at AA 559–562 in the seven fruit PLD alphas, is a highly conserved sequence of 25 AA, analogous to the DRY motif in animal G-protein-coupled receptors (Wang et al., 2006). This motif in *Arabidopsis* PLD alpha 1 was shown to bind the Gα subunit of a heterotrimeric G-protein (Zhao and Wang, 2004). Binding of Gα specifically involved the residues EKF in AtPLDα1 (corresponding to ERF in the fruit PLDs) and resulted in the inhibition of PLD activity. Addition of GTP restored enzymatic activity and inhibited binding of Gα to AtPLDα1. There is evidence that interaction of PLD alpha and G-proteins is involved in mediation of ABA signaling in plants (Ritchie and Gilroy, 1998), and conceivably could play a part in other physiologically important signaling events.

With regard to the PLD alpha catalytic sites, whereas the first HKD motif appears to be relatively isolated, the second HKD, located about 320 AAs downstream (AA 660–667 in LePLDα1; Fig. 9.12), is flanked by several motifs (including GSxN) that are important to enzyme action, all of which together define the PLD domain. Sung et al. (1997) elegantly demonstrated with a series of point mutations that both HKD motifs in human PLD1 are absolutely essential for catalytic activity; even highly conserved substitutions (e.g., R for K) inactivated the enzyme. The complexity of the PLD active site becomes apparent through several features that include substrate preference shown according to the nature of phospholipid head groups and the degree of unsaturation in the fatty acyl chains, along with the differing influence of various activating factors. PLD action involves the formation of a phosphohistidine covalent enzyme intermediate (Gottlin et al., 1998) and has been proposed to follow a ping-pong mechanism. The results of the HKD domain point mutational analyses (Sung et al., 1997) indicated that both HKD motifs in PLD must come together and form a single active site for enzyme function. This was confirmed in a study by Xie et al. (1998) showing that association of the N- and C-terminal catalytic domains of rat PLD1 is required for catalytic activity. Stuckey and Dixon (1999), on the basis of their analysis of the crystal structure of a PLD superfamily member, proposed that the histidine residue in one HKD motif forms the phosphoenzyme intermediate, histidine in the other HKD motif acts as a general acid in cleavage of the phosphodiester bond, and the pair of conserved lysine residues take part in phosphate binding.

In plant PLD alphas, flanking both sides of the second HKD domain are motifs comprising the hydrophobic amino acids isoleucine, phenylalanine, and valine. These motifs may provide the hydrophobic interaction required for fatty acid anchoring during catalysis and thus impart the fatty acyl chain specificity of the substrates hydrolyzed. Earlier studies have shown that medium-chain aliphatic alcohols and aldehydes such as hexanol and hexanal are strong inhibitors of PLD action (Paliyath et al., 1999). These components are likely to exert their inhibitory action by binding to a hydrophobic site that is essential for catalytic action. The hydrophobic motifs flanking the second HKD motif may serve as such sites. Located just before the first hydrophobic motif (shown in Fig. 9.12), there is another motif rich in basic amino acids (3 R in LePLDα1, FaPLDα1, CmPLDα1, CmPLDα2, and CsPLDα1, 2 R + 1 K in LePLDα2, and 2 R + 1 K + 1 H in LePLDα3) that is considered important for the binding of anionic lipid activators of PLD including PIP_2 and PA (Dawson and Hemington, 1967; Wang, 1999, 2002). These hydrophobic and positively charged motifs are highly conserved and present in PLD alpha, beta, and gamma isoforms. A second PIP_2-binding site in PLD gamma, located at AA 787–791 and comprising RRVRQ (Qin et al., 1997), is present in the modified sequences RKVNQ in LePLDα1 (AA 740–744), and RKVNK in

LePLDα2 and LEPLDα3 (AA 738–742). The degree of conservation declines in the other fruit PLD alphas. It should be noted that the PIP_2-binding domains in PLD alphas are, in comparison with PLD beta and gamma, deficient in one or more positively charged, basic AA, and consequently binding of PIP_2 may be reduced as noted in *Arabidopsis* PLD alpha (Qin et al., 1997; Wang, 2000).

Differential expression and subcellular distribution of various PLDs have been studied during plant development. In a study that explored these aspects using *Arabidopsis*, Fan et al. (1999) indeed observed a differential expression and subcellular distribution of various PLD classes. However, the relative roles of these classes are less clear since the PIP_2-independent activity (PLD alpha) is nearly a 1,000-fold higher than the PIP_2-dependent activity (PLDs beta and gamma). The categorization of PLD on the basis of PIP_2 dependence may be somewhat misleading since an earlier report (Qin et al., 1997) showed that all the PLD isoforms were stimulated by PIP_2, and in the order alpha > beta > gamma, when the reaction conditions for PLD alpha included 5-mM calcium and no detergent. As well, PIP_2 binding to PLD isoforms is not a reliable criterion since this binding could be highly influenced by the presence of calcium ions (Zheng et al., 2000). With the sequence and structural analogy to *Arabidopsis* PLD beta and gamma at the active site and the C2 domain, PLD alphas from tomato, strawberry, and melon may play a universal role during fruit development and ripening.

9.7 Antisense suppression of Le PLDα1 and its influence on developmental events

The role of PLD action in membrane deterioration during senescence has been studied through antisense suppression of PLD expression. Antisense suppression of PLD alpha in *Arabidopsis* resulted in retardation of ABA- and ethylene-promoted leaf senescence (Fan et al., 1997) without affecting the natural senescence of leaves. ABA and ethylene also enhanced the expression of PLD alpha. From these results, it was concluded that PLD alpha was not a direct promoter of natural senescence. However, several studies show that PLD alpha has a direct role in promoting fruit senescence. Antisense suppression of PLD alpha in Celebrity tomato fruit resulted in a decrease in PLD expression and activity in fruit during development. Even though very little transcript was detected at the mature green, orange, and red stages in the antisense PLD Celebrity fruit, phospholipase D activity was still present at these stages. This suggests that PLD may have a very low turnover rate when it is bound to the membrane, and PLD synthesized at young/intermediate stages remain functional even at the red stage. Alternatively, because the coding region of *LePLDα1* used in the antisense construct shares only 73% nucleotide identity with the coding regions of *LePLDα2* and *LePLDα3*, expression of these other two PLD alpha isogenes may not be completely suppressed in the Celebrity antisense lines, and their transcripts would not have been detected in Northern blots using the 309-bp *LePLDα1* probe at high stringency. If in fact PLD alpha enzyme has a low rate of turnover, PLD expression has to be reduced at an early stage of fruit development for effective inhibition of PLD via antisense suppression. During ripening, a significantly lower level of PLD activity was maintained in the transgenic Celebrity fruit, showing that natural senescence process was retarded, which was translated into increased firmness in these fruits. Thus, fruits may differ in their pattern of senescence, and the relative role of PLD may differ between fruits and leaves. As well, the observation

that PLD beta and gamma isoforms could not replace the decreased PLD alpha activity induced by antisense suppression may have arisen because of the naturally low PLD beta and gamma activity (nearly 1,000-fold) in *Arabidopsis* (Fan et al., 1999).

9.7.1 Generation of antisense PLDα1 tomato plants

Transformation of "Celebrity" tomatoes was conducted through *Agrobacterium*-mediated transfer of the antisense construct to excised cotyledons. For this, a binary vector designated pBIN-mgfp5-ER (Pinhero et al., 2003) was used. The *GFP* (cDNA sequence for green fluorescent protein) was dissected out and a 2,427-bp PLD cDNA was introduced in the antisense orientation between a *Bam* H I site and a *Sac* I site. The control of transcription initiation and termination was achieved by a CaMV 35S promoter (constitutive) and an NOS terminator, respectively. The transformed cotyledons were grown in vitro in the presence of kanamycin. Kanamycin-resistant calli and seedlings that possess a positive transformation were passed through two additional generations in the presence of kanamycin, and further transferred and grown in a regular MS rooting medium. Rooted seedlings were further transferred to sterile perlite and grown in a greenhouse. The presence of the *NPT* gene (kanamycin resistance) in transformed plants was confirmed by PCR amplification of the DNA using primers specific for the *NPT* gene, which resulted in the amplification of a 700-bp *NPT* gene segment. The transformed plants were propagated clonally to eliminate the possibility of the loss of antisense PLD cDNA in subsequent generations. The cotyledons were also transformed with the vector alone and plants regenerated. These plants could serve as controls to eliminate the physiological repercussions of the transformation process. The transformed plants were always compared with control plants that originated from cotyledons through the tissue culture procedure.

9.7.2 Characteristics of Celebrity antisense PLDα1 tomatoes

The transformation and regeneration efficiency of Celebrity tomatoes was very low ($\leq$2%). The transformed plants were very vigorous in vegetative growth. Many of the transformed plants produced flowers as usual, but did not set fruit. The plants transformed with the vector alone showed slow vegetative growth and did not set flower. Among several antisense PLD strains grown, one strain produced normal flowers and set fruits. The fruits from the antisense PLD plants were smaller than the control fruits. The pictures of a set of control (C) and antisense PLD (T) Celebrity tomatoes at harvest are given in Fig. 9.13. During subsequent storage for 2 weeks, the control fruits developed wrinkles indicative of senescence and dehydration (Fig. 9.13), whereas the fruits from the antisense PLD plants appeared relatively normal. The loss of fresh weight was more or less identical in fruits from control and transgenic plants. Thus, the appearance of wrinkles may be indicative of a loss of structural integrity. The transgenic fruits were also firmer and possessed a higher level of red pigmentation (Table 9.4) as indicated by a higher level of lycopene, a^+ (red) value and a^+/b^+ (red/yellow) color ratio. The antisense PLD fruits also possessed an increased level of soluble solids and vitamin C (Table 9.4). A cross section of control and transgenic fruits is shown in Fig. 9.13 (C, T), respectively. The control fruits are multilocular, whereas the transgenic fruits are bilocular.

Table 9.4 Color and firmness of control and phospholipase D antisense Celebrity tomatoes

Quality parameters	Control tomato fruits	Antisense tomato fruits
Firmness (N/mm)	4.97 ± 0.59 a	5.99 ± 1.03 b
Fruit weight (g)	95.14 ± 36.56 a	34.87 ± 21.57 b
Red intensity (a+)	29.60 ± 0.70 a	31.70 ± 1.67 b
Yellow intensity (b+)	17.22 ± 0.06 a	18.42 ± 2.65 a
Vitamin C (mg/100 g)	3.9 ± 0 a	10.4 ± 0.016 b
Lycopene (mg/100 g Juice)	12.19 ± 0.09 a	17.92 ± 0.43 b
Nonsoluble solids (%)	3.54 ± 0.05 a	5.15 ± 0.03 b
Soluble solids (%)	5.90	6.60

Nine fully ripe fruits from each set were harvested and used for color and firmness measurements. Color was measured by a Minolta Chromameter (CR 300) at two spots in the midlocular region of tomatoes. The values are mean ±SE from 18 readings. Firmness was measured by a texture analyzer. The values are mean ±SE from 18 readings, expressed in newton. The pressure was measured using a hand-held probe with an 8-mm probe tip. Soluble solids were measured using a hand-held refractometer.

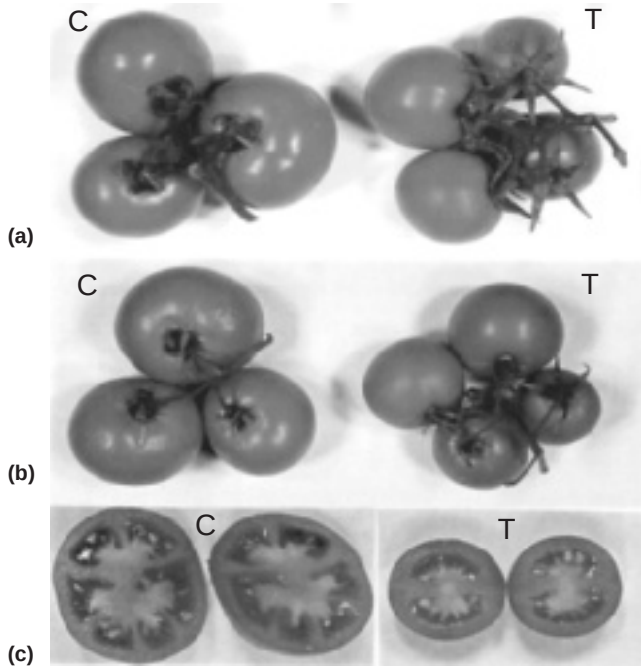


Fig. 9.13 Morphological characteristics of control and antisense PLD tomatoes. (a) Celebrity control (C) and antisense PLD (T) tomatoes at the time of harvest. (b) Celebrity control (C) and antisense PLD (T) tomatoes after 2 weeks of storage at 22°C. (c) A cross section of Celebrity control (C) and antisense PLD (T) tomatoes. (Reproduced with permission from Pinhero et al., 2003.)

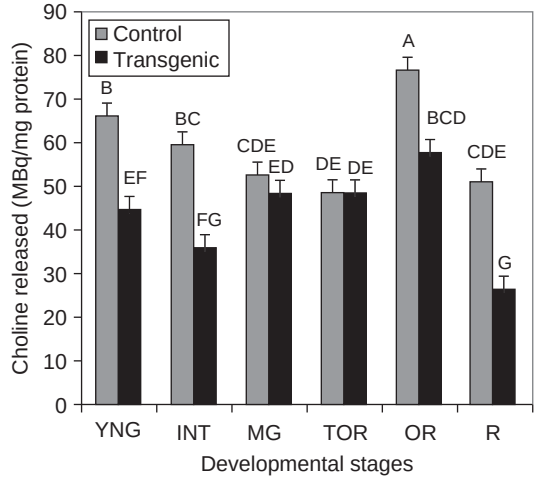


Fig. 9.14 PLD activities observed in the total homogenates of control (red) and antisense PLD (yellow) Celebrity tomato fruits. The analysis was conducted at different developmental stages: young (YNG), intermediate (INT), mature green (MG), turning orange (TOR), orange (OR), and red (R). PLD activity at young, intermediate, orange, and red stages are significantly lower in the antisense PLD tomatoes as compared to the controls. The histograms with different alphabets indicate values that are significantly different at $p \leq 0.05$. (Reproduced with permission from Pinhero et al., 2003.)

9.7.3 PLD activity in control and transgenic tomato fruits

PLD activity was also estimated in fruits from "Celebrity" plants transformed with antisense PLD cDNA during developmental stages (Fig. 9.14). The fruits of Celebrity control plants possessed significantly higher levels of PLD activity at the young, intermediate, orange and red stages and were similar at the mature green and turning orange stages. Perhaps, this feature is physiologically translated into increased firmness in fruits of antisense PLD plants.

9.7.4 Changes in PLDα1 mRNA during development

The expression of *PLD* gene during fruit development was analyzed by Northern blotting. For this, a DIG-labeled 309-bp RNA segment that corresponds to the carboxyl terminal region of PLD alpha 1 cDNA was used as the probe. In both cherry tomato fruits and Celebrity fruits, this probe hybridized with RNA having a length of nearly 2,500 bp, the anticipated size of LePLD alpha 1 mRNA. The pattern of expression of *LePLD* alpha 1 during development of cherry tomato fruits is shown in Fig. 9.15a. The RNA samples were analyzed at the young, intermediate, mature green, turning orange, orange, and red stages. The expression of *PLD* increased during development and attained a maximum during mature green and turning orange stages, nearly in parallel with the pattern of changes observed in the activity profile and PLD protein levels (Pinhero et al., 2003). Thus, there is a clear correlation between increase in PLD activity, PLD protein levels, and *PLD* gene expression at various stages of cherry tomato fruit development.

A similar analysis of *LePLD*α1 gene expression was conducted using Celebrity tomatoes at young, intermediate, mature green, turning orange, orange, and red stages. Normal and

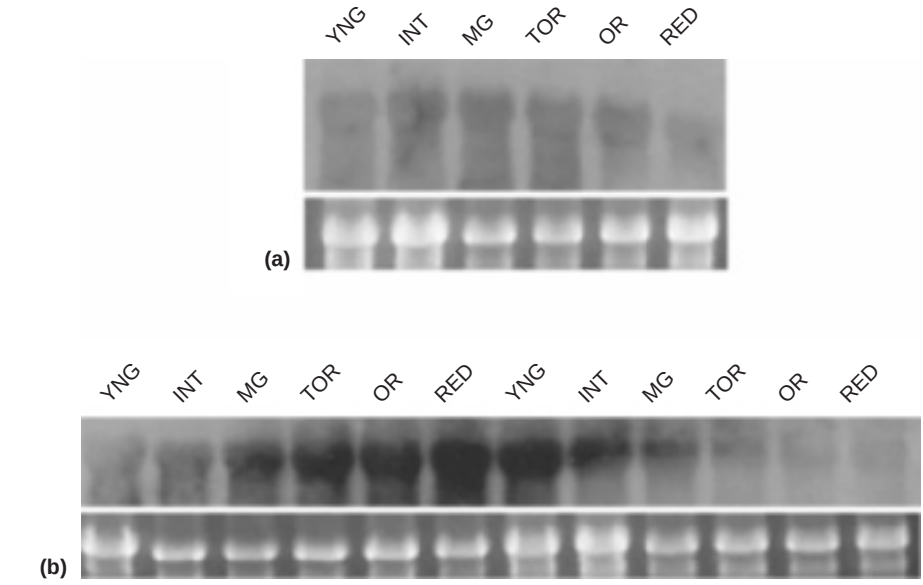


Fig. 9.15 Analysis of *PLD* gene expression in cherry tomato fruits, control and antisense Celebrity fruits, during development. The stages analyzed include young (YNG), intermediate (INT), mature green (MG), turning orange (TOR), orange (OR), and red (RED). (a) Northern blot analysis of cherry fruit RNA using DIG-labeled RNA probe synthesized for PLD alpha (top profile). The bottom profile indicates the 18S rRNA stained with ethidium bromide to indicate loading variability of RNA. (b) Northern blot analysis of RNA samples from Celebrity tomatoes. The top profile shows the intensity of binding with DIG-labeled RNA probe for PLD alpha. The bottom profile shows staining intensity of 18S rRNA with ethidium bromide, indicative of loading variability. The first six lanes from left to right are representative of RNA samples from young, intermediate, mature green, turning orange, orange, and red stages of control Celebrity tomato fruits. The next set of six lanes (7–12) is representative of RNA samples from equivalent developmental stages of the antisense PLD Celebrity tomato fruits. (Reproduced with permission from Pinhero et al., 2003.)

antisense PLD Celebrity tomatoes were used in these analyses. A northern blot of RNA from developing Celebrity tomatoes is shown in Fig. 9.15b. As with cherry tomato fruit RNA, the *PLD* alpha mRNA probe showed hybridization with a 2,500-bp RNA. *PLD* mRNA levels increased during development of normal fruits (Fig. 9.15b; lanes 1–6 from the left depicting young, intermediate, mature green, turning orange, orange, and red stages) and peaked at the orange/red stages. By contrast, in the fruits from antisense PLD Celebrity plants, the highest level of expression was observed at the young and intermediate stages (Fig. 9.15b; lanes 7 and 8 from the left). *PLD* expression progressively declined during further development, and only trace levels of hybridization could be detected at the mature green, turning orange, orange, and red stages (lanes 9, 10, 11, and 12 from the left). This result conclusively showed that *PLD* alpha expression was reduced during fruit ripening by the introduction of antisense *PLD* alpha cDNA in Celebrity tomatoes.

9.7.5 Antisense suppression of *LePLDα2* in "Rutgers" tomato fruit

A study of *LePLDα2* antisense suppression in the Rutgers tomato cultivar took a somewhat different approach from that of the prior investigation of *LePLDα1* antisense suppression in

"Celebrity" tomatoes. In particular, with the aim of minimizing possible effects of PLD alpha gene disruption on plant growth and development, the fruit-specific *E*8 promoter was used instead of the constitutive *CaMV35S* promoter. The *E*8 promoter is responsive to ethylene, but high-level expression in tomato fruit appears to be regulated by an ethylene-independent, ripening-related *cis* element (Deikman et al., 1998). Aside from fully developed fruit tissues, *E*8 is expressed mainly in anthers of mature, stage 4 flowers. An antisense construct of *LePLDα2* was cloned into the binary vector pBI121 (with 35S and GUS excised) for use in *Agrobacterium*-mediated transformation of tomato cotyledon explants. The entire construct included a copy of the npt II (neomycin phosphotransferase) antibiotic-resistance gene under control of the *NOS1* (nopaline synthase) promoter and terminator, and a 2.0-kb fragment of the *LePLDα2* cDNA (nucleotides 880–2,846, constituting 70% of the coding region plus the 3′-UTR) in antisense orientation regulated by the *E*8 promoter and *NOS1* terminator.

Following selection based on kanamycin resistance, 25 putative antisense *LePLDα2* transformants were identified by DNA gel blot (Southern) analysis using leaf tissue genomic DNA and probes for npt II and *E*8. Of these, plants representing 16 lines were raised to maturity, and pericarp tissue from mature green fruit (40 days after pollination) was tested for suppression of *LePLDα2* by semiquantitative RT-PCR using isolated total RNA. The Ambion QuantumRNA™ quantitative RT-PCR kit was used to determine levels of *LePLDα2* cDNA relative to control 18S cDNA. The amplified *LePLDα2* and 18S cDNA fragments produced by RT-PCR were separated on agarose gels and quantified by densitometry measurements after staining with ethidium bromide. Among the sixteen lines tested, four showed clear suppression of *LePLDα2*, whereas three surprisingly exhibited overexpression of the gene. The four *LePLDα2* suppressed lines (designated 8-4-A, 10-3-B, 10-5-C, and 10-6-C), plus line 8-2-E that showed the highest level of *LePLDα2* overexpression, were propagated through two subsequent generations by self-fertilization for further analyses of PLD alpha gene expression and activity, as well as fruit physiology and quality attributes.

9.7.6 Levels of *LePLDα* transcripts in fruit of *LePLDα2* antisense lines

LePLDα2 transcript abundance in pericarp of wild type and antisense transgenic fruit harvested 40 days after pollination (DAP) was determined by both RNA gel blots (Northern blots) and the semiquantitative RT-PCR 18S competimer system (Ambion QuantumRNA) described earlier (Fig. 9.16). For the Northern blots, a 646-bp segment of the *LePLDα2* cDNA coding region (nucleotides 1,080–1,725) was used as the radiolabeled probe, whereas in the RT-PCR experiments a 0.6-kb cDNA fragment of *LePLDα2* (coding region nucleotides 735–1,323) was amplified. The two methods gave similar results, and averaging the two values, transcript levels in the four suppressed lines ranged from about 20 to 45% of that in wild-type controls, and transcript in line 8-2-E was roughly 75% higher than that in wild type.

Concerning possible suppression or overexpression of the other two tomato PLD alpha isogenes, because of the high percentage of sequence identity between *LePLDα2* and *LePLDα3* (91% in the open reading frame), it seemed likely that *LePLDα2* antisense would also affect expression of the *LePLDα3* isogene. However, perhaps because the 3′-UTR was included in the 2-kb antisense *LePLDα2* fragment, this does not appear to be the case. Semiquantitative RT-PCR analysis indicated that in pericarp tissue of fruit harvested at 40 DAP there was little if any reduction (or increase) in *LePLDα3* transcript in the five *LePLDα2*

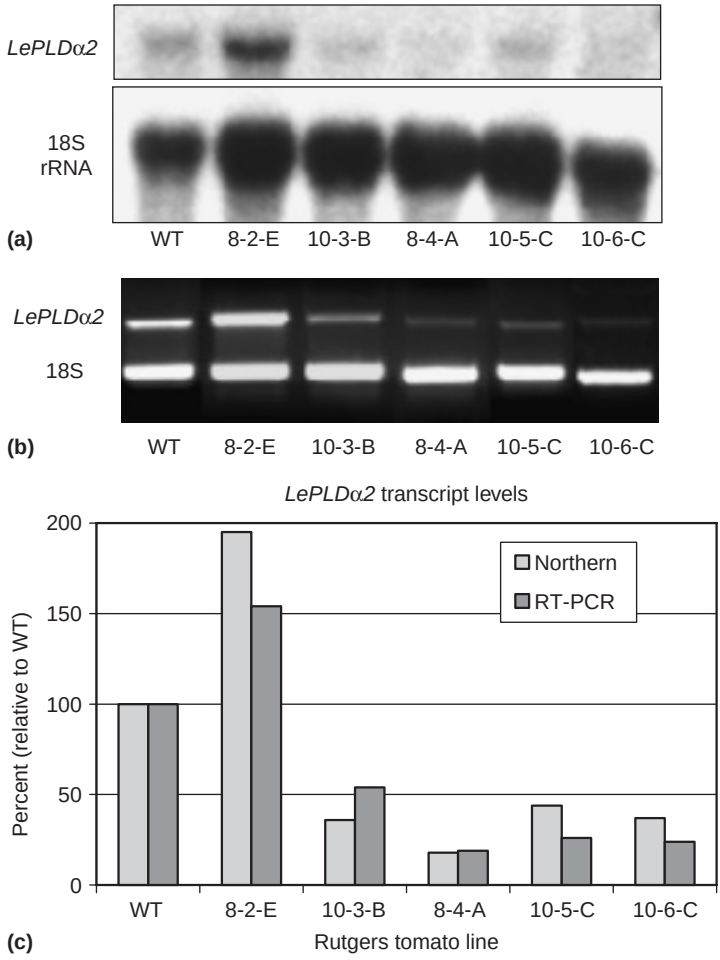


Fig. 9.16 Comparison of RNA gel blot and semiquantitative RT-PCR analyses of *LePLDα2* transcript levels in outer pericarp tissue of mature green fruit from wild type (WT) and five *LePLDα2* antisense transgenic lines (8-2-E, 10-3-B, 8-4-A, 10-5-C, and 10-6-C) of "Rutgers" tomato. RNA gel blot analysis (a) was performed using ca. 20 μg per lane total RNA probed with a 645-bp fragment of the *LePLDα2* cDNA open reading frame (nucleotides 1,080–1,725). The blot was then stripped and hybridized with a probe for 18S rRNA to evaluate loading of total RNA. The QuantumRNA quantitative RT-PCR kit (Ambion) was used to determine levels of *LePLDα2* cDNA relative to the control 18S cDNA (b). Ethidium bromide-stained gels of the RT-PCR products were used for densitometric measurements of the amplified *LePLDα2* and 18S cDNA fragments. The bar graph (c) shows the relative levels of *LePLDα2* transcript in the six tomato lines determined by the two methods, with WT values set at 100%.

antisense transgenic lines (Fig. 9.10b). Considering these results, it is very unlikely that *LePLDα1* expression is altered in the *LePLDα2* antisense lines, but this was not tested.

9.7.7 Total PLD alpha activity in fruit of *LePLDα2* antisense lines

Pericarp tissue from fruit of "Rutgers" wild type and *LePLDα2* antisense transgenic lines harvested at four ripening stages (mature green, breaker, turning, and pink/orange) was

lyophilized and then used to prepare acetone powders as described by Jandus et al. (1997). Aliquots of the soluble supernatant (200 μL of 3 mL) derived from a homogenate of 0.1 g of the acetone powder were used in assays of total PLD alpha activity as reported by Whitaker et al. (2001), with the exception that 800 nmol of soybean PC was included as substrate in each assay. At the end of incubation (5–40 min) with gentle shaking at 30°C, the reaction was terminated by addition of chloroform/methanol, 2:1 and vortexing. The remaining substrate (PC) and hydrolysis product (PA) were extracted in the chloroform phase, separated by thin-layer chromatography, and quantified by ashing and assay of phosphate (Ames, 1966). This method was used previously to demonstrate PLD alpha activity of the enzyme produced by expression of the *LePLDα2* cDNA open reading frame in *E. coli* (Whitaker et al., 2001).

The results of a series of assays with the soluble PLD preparations from fruit of wild-type and *LePLDα2* antisense lines 8-2-E, 10-6-C, 10-5-C, and 10-3-B at four ripening stages are shown in Fig. 9.17. Values in the bar graph are expressed as percent hydrolysis of the PC substrate during a 15-min incubation. As expected, wild-type control samples boiled for 5 min and then cooled prior to assay were devoid of PLD activity. For wild-type fruit, PC hydrolysis nearly doubled between the mature green and turning stages, then dropped off somewhat by the pink stage. Antisense line 8-2-E, which exhibited substantially higher levels of *LePLDα2* transcript than WT when mature green (Fig. 9.17), also showed a faster rate of PC hydrolysis at this early stage of ripening. Thereafter (breaker through pink), the percent PC hydrolysis in 15 min was roughly equivalent in 8-2-E and wild type. Although PLD alpha activity at the four ripening stages was rather variable among the three *LePLDα2* antisense-suppressed lines tested, each showed a reduced level of activity commensurate with the reduction in *LePLDα2* transcript (Fig. 9.16). It is notable that PLD alpha activity was almost nil in mature green fruit of 10-6-C and 10-5-C, whereas in 10-3-B activity was

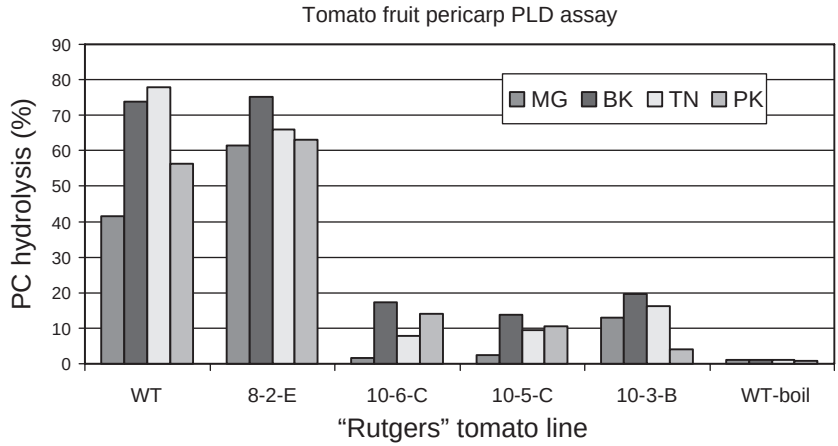


Fig. 9.17 Assay of soluble PLD alpha activity in acetone powder prepared from outer pericarp tissue of "Rutgers" tomato fruit harvested at four ripening stages (MG, mature green; BK, breaker; TN, turning; PK, pink/orange). Data are presented for fruit from five lines, including the wild type (WT) and four *LePLDα2* antisense transgenic lines numbered 8-2-E, 10-6-C, 10-5-C, and 10-3-B. The bars indicate the percent hydrolysis of 800 nmol of soybean PC to phosphatidic acid during incubation for 15 min at 30°C. Boiled wild-type control samples (WT boil) were also included in the assay. Relative to the wild type, antisense lines 10-6-C, 10-5-C, and 10-3-B each exhibited about 50–80% suppression of *LePLDα2* expression at the mature green stage of fruit development, whereas line 8-2-E showed an unexpected 50% increase in *LePLDα2* transcript.

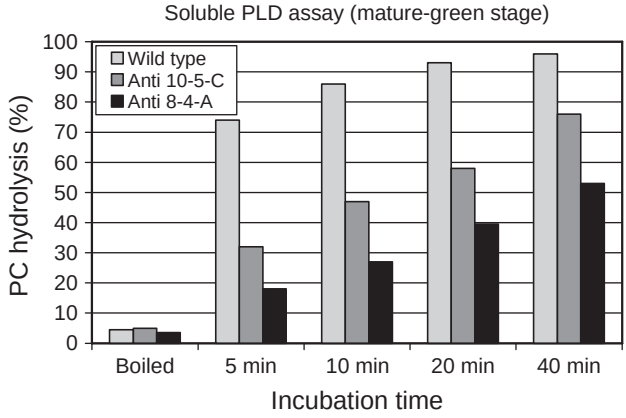


Fig. 9.18 Assay of soluble PLD alpha activity in acetone powder prepared from outer pericarp tissue of "Rutgers" tomato fruit harvested 40 days after pollination (mature green stage). Data are presented for fruit from three lines, including the wild type and two *LePLDα2* antisense transgenic lines numbered 10-5-C and 8-4-A. The bars indicate the percent hydrolysis of 800 nmol of soybean PC to phosphatidic acid (PA) during incubation at 30°C for 5, 10, 20, or 40 min. Methods were the same as those used in the assays at different ripening stages (Fig. 9.17) with the exception that soluble PLD preparations were twofold more concentrated (0.2 g acetone powder used). Boiled control samples from each line were also included in the assay. Relative to the wild type, antisense lines 10-5-C and 8-4-A exhibited, respectively, about 65 and 80% lower levels of *LePLDα2* transcript at the mature green stage of fruit development.

five- to tenfold higher at the mature green stage. Again, this is in accord with the *LePLDα2* mRNA levels in fruit harvested at 40 DAP (mature green).

In addition to assays of PLD alpha activity in fruit of wild type and *LePLDα2* antisense lines at various stages of ripening, a time-course analysis of PLD alpha activity with incubation times ranging from 5 to 40 min was also performed, comparing mature green fruit of wild type and two antisense lines, 10-5-C and 8-4-A (Fig. 9.18). For these time course assays, the concentration of the enzyme preparations was doubled such that about 73% of the PC substrate was hydrolyzed to PA in 5 min by the wild-type PLD preparations. With longer incubations, the percent of PC hydrolysis with the wild-type enzyme approached 100 asymptotically, reaching a value of about 96% after 40 min. PLD preparations from fruit of the two *LePLDα2* antisense lines, on the other hand, gave an essentially linear increase in percent PC hydrolysis over the span of 5–40 min. After a 40-min incubation period, the extent of PC hydrolysis in the assay for antisense line 10-5-C (about 76%) slightly exceeded that observed after 5 min in the wild-type assays, indicating a roughly eightfold lower level of PLD alpha activity in the transgenic fruit. The rate of PC hydrolysis was considerably slower with enzyme preparations from fruit of the 8-4-A antisense line, and by extrapolation, the level of PLD alpha activity was about 30-fold lower in 8-4-A compared with wild-type fruit.

9.7.8 Influence of *LePLDα2* antisense suppression on fruit physiology

Ripening, both on the plant and after harvest was delayed and/or protracted in *LePLDα2* antisense compared with wild-type fruit. Surprisingly, however, this general effect on fruit physiology was observed in the *LePLDα2* overexpressing line, 8-2-E, as well as in the antisense suppressed lines. Relative to the wild-type fruit, both the rise in ethylene production

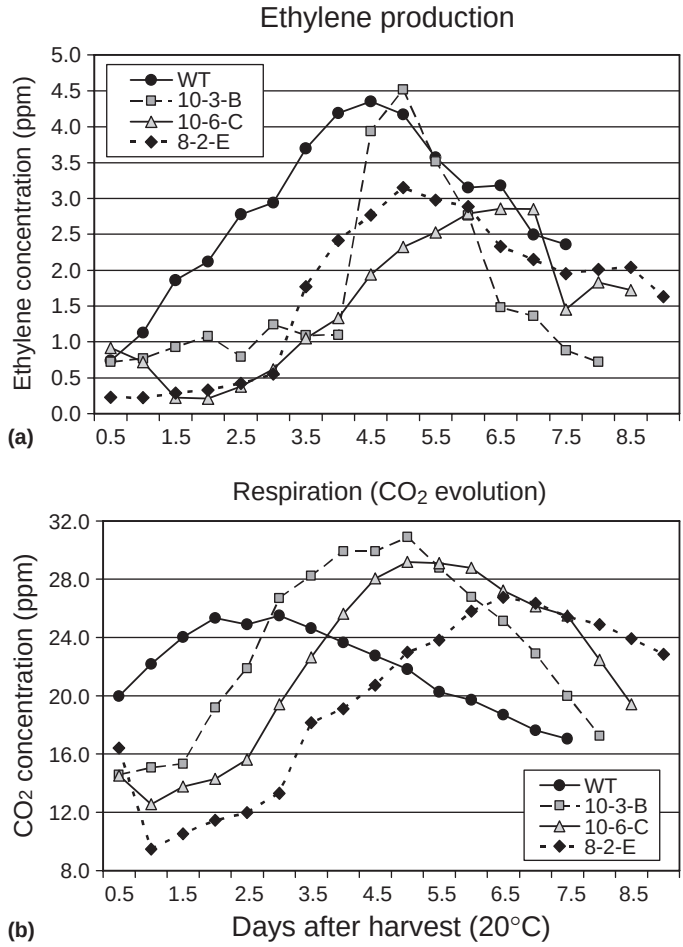


Fig. 9.19 Patterns of (a) ethylene production and (b) CO_2 evolution (respiration) in fruit of wild type (WT) and *LePLDα2* antisense transgenic lines (10-3-B, 10-6-C, 8-2-E) of "Rutgers" tomato harvested at 40 days after pollination (mature green stage in WT). Individual fruit of similar size and weight (6–8 per line) were sealed in 1-L jars connected to a flow through automated system that measured ethylene and CO_2 concentrations by gas chromatography-flame ionization detection every 6 h for up to 9 days.

and the increase in respiratory CO_2 evolution associated with early stages of tomato ripening were delayed in fruit of the three antisense lines, albeit to differing degrees (Fig. 9.19). Fruits were harvested at 40 DAP, generally corresponding to late mature green stage in the wild type. The delay in peak ethylene production was greatest for the antisense suppressed line 10-6-C, and the maximum level of ethylene was lower in both 10-6-C and 8-2-E than in 10-3-B and wild type (Fig. 9.19a). Although the delay in ethylene production was similar for lines 10-3-B and 8-2-E, fruit of the former showed an unusual rapid rise and decline, peaking at about 5 days after harvest. It was evident that the wild-type fruit had already begun the respiratory climacteric at harvest, whereas fruit of antisense lines 10-3-B, 10-6-C, and 8-2-E entered the climacteric at successively later times during postharvest ripening at 20°C (Fig. 9.19b). Maximum rates of CO_2 evolution were somewhat higher in fruit of

10-3-B and 10-6-C than in those of wild type and 8-2-E. Overall, the relative effects on ethylene and CO_2 production in the fruit of antisense lines 10-3-B and 10-6-C were consistent with their respective levels of *LePLDα2* suppression at 40 DAP (Fig. 9.10a). However, it is hard to reconcile the delays in ripening and maximum ethylene and CO_2 production in fruit of the antisense line 8-2-E because they exhibit both overexpression of *LePLDα2* and high levels of PLD alpha activity at 40 DAP (Figs. 9.10a and 9.19). Hypothetically, delayed ripening could be a secondary effect of transformation. Unfortunately, the empty vector lines (including *E8* without the antisense *LePLDα2* insert), which were intended as transformation controls, did not yield enough viable seed for further propagation.

9.7.9 Quality attributes of ripened *LePLDα2* antisense transgenic fruit

Evaluation of quality in fruit harvested at breaker plus 7 days was limited to three *LePLDα2* antisense lines (10-3-B, 10-6-C, and 8-2-E) compared with wild type, and three simple parameters, including fresh weight, red color, and whole fruit firmness (Fig. 9.20). Fruit size and weight varied considerably within each line, but the mean fresh weight was not markedly different among the four lines, ranging from about 122 to 175 g (Fig. 9.20a). Mean fresh weight of fruit from all three transgenic lines was at least slightly greater than that for wild-type fruit, and fruit weight for line 10-3-B was significantly greater than that for both 10-6-C and wild type at the 5% confidence level. The positive CIE a* values, which are a measure of "redness," were quite similar for fruit from all four lines (Fig. 9.20b). The values were highest for 10-3-B and 8-2-E, and lowest for 10-6-C, but the small differences were not significant. Mean whole fruit firmness, measured by flat plate compression analysis, was closely similar for wild type and antisense suppressed lines 10-3-B and 10-6-C (Fig. 9.20c). In contrast, fruits of the antisense line 8-2-E, with anomalous overexpression of *LePLDα2* at 40 DAP (Fig. 9.10a), were significantly firmer ($p > 0.05$) than those from the other three lines. This is in accord with the delayed, relatively low-level ethylene production, and much delayed (ca. 5 days relative to wild type) respiratory maximum exhibited by fruit of this line (Fig. 9.19).

9.8 PLD in signal transduction

Molecular events resulting from ethylene perception by the receptor and changes in gene expression are still being elucidated. Ethylene receptors belong to the hybrid kinase type (*ETR1*, *ETR2*, *EIN4*) or histidine kinase type (*ERS1*, *ERS2*) (Chang and Stadler, 2001). Signal reception results in the phosphorylation of a histidine residue in the transmitter domain of the two-component receptor system. The phosphate is further transferred to the receiver domain through a phosphorelay mechanism (D'Agostino and Kieber, 1999). Downstream events in the activation of gene expression proceed through MAP kinase cascade and activation of transcription of *ERF1* (EREBP—ethylene response element-binding protein). *ERF1* binds to the GCC-box promoter elements to activate ethylene-responsive gene expression. Several genes show increased expression in response to ethylene treatment of tomatoes within 15 min and include those for intermediates of signal transduction pathways (*CTR1*) and other transcription regulator proteins (Zegzouti et al., 1999). Several details of these events are yet to be worked out (Chang and Stadler, 2001; Lohrmann and Harter, 2002). A recent study shows that ethylene signal transduction can proceed in parallel pathways,

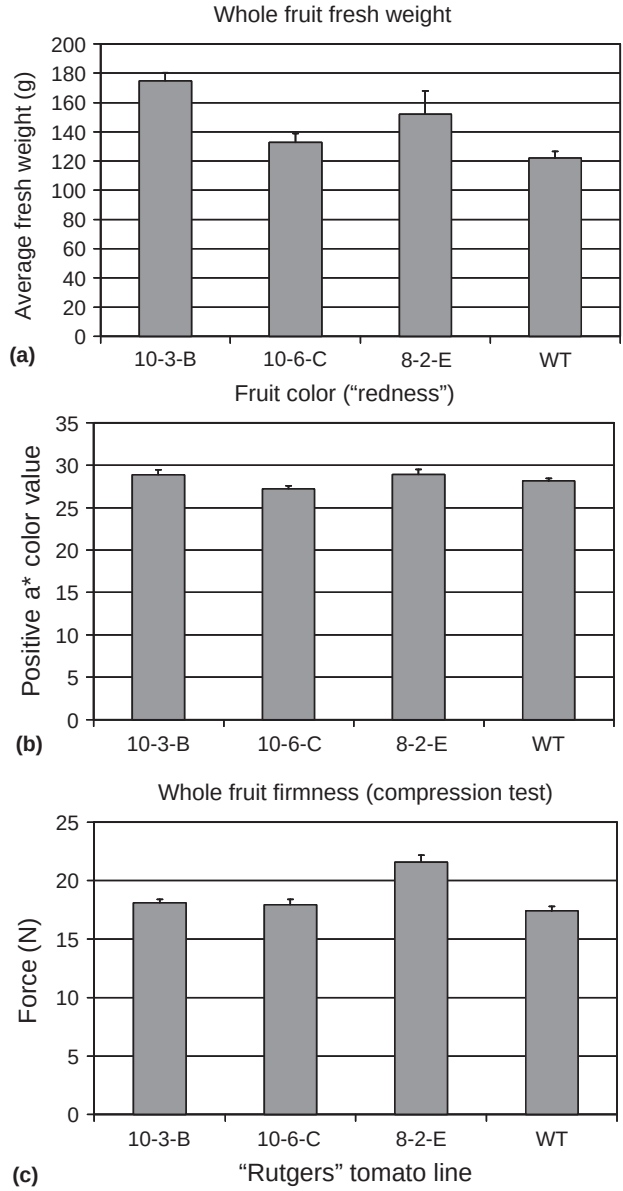


Fig. 9.20 Mean fresh weight (a), a* (red) color value (b), and firmness (c) of whole fruit from wild-type (WT) and three *LePLDα2* antisense lines harvested 7 days after the breaker stage. Error bars indicate SEM, with $n \geq 60$ for (a) and (c), and ≥ 30 for (b). Red color evaluation was based on CIE a* values (three equatorial measurements per fruit) obtained with a Minolta CR-300 colorimeter. Firmness was determined by flat plate compression of whole fruit at 2 mm/s to a depth of 5 mm performed at three equatorial sites using a Stable Microsystems Texture Analyzer (Surrey, England, United Kingdom).

originating from different receptors (ETR1, ETR2) and potentially interacting with one another, affecting internodal elongation and abscission independently from fruit ripening (Whitelaw et al., 2002).

Results from several years of study in different laboratories enable us to propose a model for the signal transduction pathway between ethylene perception and the induction of senescence at the membrane level. Since calcium and calmodulin appeared to be involved in enhancing membrane deterioration (Paliyath et al., 1987; Paliyath and Thompson, 1987), the possible link between ethylene perception and phosphatidylinositol metabolism was investigated in carnation flowers based on the hypothesis that phosphorylation of PI to PIP_2, phospholipase C action on PIP_2-generating inositol trisphosphate (IP_3), and IP_3-mediated calcium release from the endoplasmic reticulum, were potentially the sequence of events in the signal transduction pathway. When exposed to ethylene, fully open carnation flowers showed visible symptoms of senescence within 6 h of treatment. Carnation flowers were incubated with radiolabeled phosphorus and treated with ethylene. The incorporation of radiolabel into the phospholipid fraction was followed soon after treatment. If there were a specific turnover in any phospholipids in response to ethylene, then there would be a specific enrichment of the radiolabel in that phospholipid (based on phospholipid phosphate). None of the phospholipids including phosphatidylinositol showed any significant enrichment in response to ethylene treatment, suggesting that phospholipid turnover, if it occurred, was restricted to sites close to the ethylene receptor, and may occur in very small amounts not quantifiable by the available techniques. As well, with a relatively low efficiency of calcium release by IP_3 and a very low phospholipase C activity, the proposed hypothesis was not supported. The recent results on the molecular properties of PLD enable us to propose an alternate mechanism that may mediate the ethylene signal transduction pathway (Fig. 9.21). Studies on animal systems show that PI-3-kinase is activated by receptor tyrosine kinases in response to primary stimuli, which leads to the production of PI (3,4)-bisphosphate and PI (3,4,5)-trisphosphate on the inner leaflet of the plasma membrane (Blomberg et al., 1999). These anionic domains are believed to be the anchoring sites for enzymes in the signal transduction pathway, such as phospholipase C, which possesses a PH superfold (plextrin homology domain). The presence of the C2 domain in PLD (analogous to the PH superfold) provides the structural feature necessary for the electrostatic binding of PLD to anionic sites created in the membrane in response to primary stimuli. Recent evidences suggest that PI is converted to its phosphorylated forms in response to primary stimuli through PI kinases (Heilman et al., 2001). Such anionic microdomains in the membrane could provide the anchoring region for PLD even in the absence of an increase in cytosolic calcium (Zheng et al., 2000). Generation of anionic microdomains specifically in the inner leaflet of the plasma membrane could generate a voltage across a localized region of the plasma membrane (hyperpolarization), and may open voltage-sensitive calcium channels, thus increasing the cytosolic calcium levels (Roberts and Tyerman, 2002). Since the binding of PLD to anionic domains is reversible and dependent on calcium (high micromolar calcium reverses binding) (Zheng et al., 2000), such binding may serve as an on/off switch regulating calcium release, through catabolism of phosphorylated phosphatidylinositols (depolarization) (Paliyath et al., 1995). A recent report also suggests that cyclic nucleotide-gated nonselective cation channels (voltage independent) may be involved in programmed cell death in *Arabidopsis* (Kohler et al., 2001). PLD may bind to other domains of the membrane enriched in phosphatidylcholine/phosphatidylethanolamine, since this binding

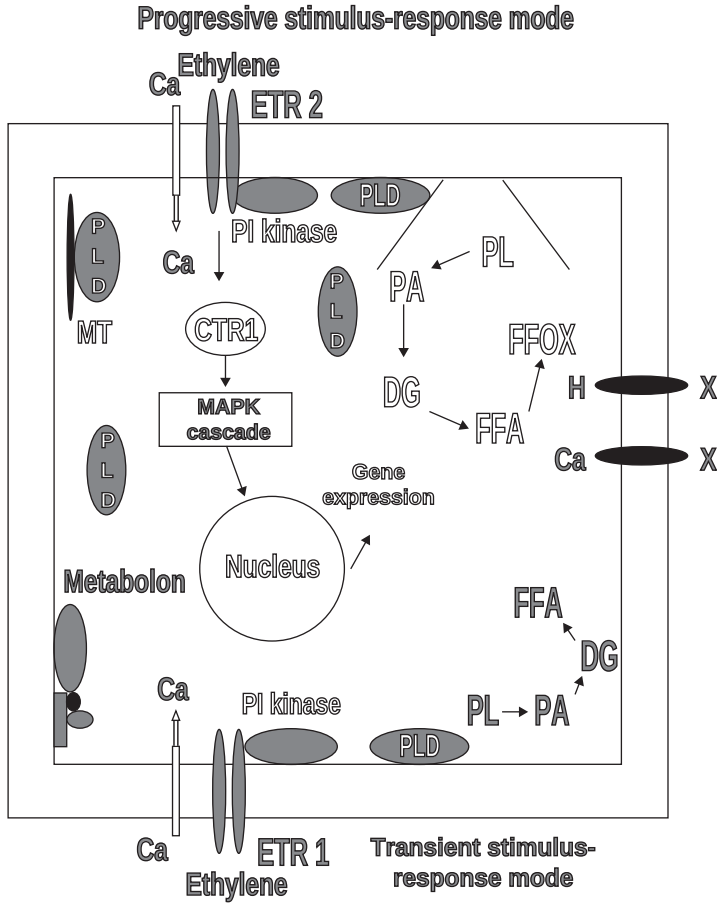


Fig. 9.21 A model describing potential events involved in the ethylene signal transduction pathway indicating the role of PLD. Ethylene binding to the receptor is proposed to activate a PI kinase that in turn results in the generation of a voltage difference across the membrane. Voltage-sensitive calcium channels may open in response to the voltage difference, increasing the cytosolic calcium level. PLD can bind to the membrane at the anionic domains created by PI-kinase activity or in response to the increased cytosolic calcium levels. In the progressive stimulus-response model, the hormonal stimulation continues ultimately inactivating calcium and proton pumps (H-X, Ca-X). Excessive cytosolic calcium would ultimately cause senescence. In the transient stimulus-response model, cytosolic calcium level is maintained at homeostatic levels. Lipid catabolites are recycled back to phospholipids. ETR1 and ETR2 are designation of receptors with potentially distinct function. CTR-1 is a protein kinase that acts downstream from the receptor. PLD could exist as a metabolon (left bottom) in combination with phosphatidate phosphatase, calmodulin, and lipolytic acyl hydrolase, or even bound to microtubules (MT) (DG, diacylglycerol; FFA, free fatty acid; FFOX, fatty acid oxidation products; PA, phosphatidic acid; PL, phospholipid).

is promoted by calcium. Under normal conditions of growth and development, this switch would be tightly regulated (transient stimulus-response model) due to cross talk with other signals (Lohrmann and Harter, 2002). After the initiation of a programmed senescence event (progressive stimulus-response model), such tight regulation could be lost and would eventually lead to high cytosolic calcium and lowered cytosolic pH, as the calcium ATPase and proton ATPase are inhibited, respectively (Paliyath and Thompson, 1988; Paliyath et al.,

1997). The sequences of potential events in the signal transduction pathway are depicted in Fig. 9.21.

In conclusion, our series of recent studies have clearly demonstrated a role of various PLD alphas in the processes of fruit development and ripening. The *LePLDα* cDNAs that have been isolated from various fruits appear to be unique with respect to their developmental regulation and the putative properties of the encoded protein, especially in relation to potential calcium-binding motifs in the C2 domain and the phospholipase D active site domains. Further studies are in progress to understand the biochemical mechanism of interaction of various motifs in the overall regulation of PLD.

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Chapter 10

Phospholipase D Inhibition Technology for Enhancing Shelf Life and Quality

Gopinadhan Paliyath and Jayasankar Subramanian

10.1 Introduction

The role of plasma membrane in the preservation of cell structure and integrity was discussed in the previous chapter. Phospholipase D (PLD) is the key enzyme which initiates a series of catabolic cascades that lead to the eventual deterioration of the membrane. The deterioration of the membrane is a part of the development of the organoleptic quality of the fruits during ripening. However, uncontrolled progression of lipid degradation can reduce the life of fruits, vegetables, and flowers. Previous technologies for the preservation of horticultural produce quality were based on the regulation of ethylene biosynthesis (e.g., Retain, Valent Biosciences, a trade name for the ethylene biosynthesis inhibitor aminoethoxyvinylglycine) or its action (e.g., 1-MCP, AgroFresh Inc.). Inhibition of cell wall degradation using calcium chloride was another approach that increased the firmness in fruits such as apple. Biotechnological approaches for the inhibition of ethylene biosynthesis using antisense ACC synthase have been found to be an effective approach to enhance fruit firmness in apple fruits (Cornell University College of Agriculture and Life Sciences, 2002; http://www.nysaes.cornell.edu).

The process of membrane deterioration during senescence has been well documented (McKersie et al., 1978; Paliyath and Droillard, 1992). The preservation of membrane integrity is critical to the maintenance of membrane compartmentalization. As well, membranes are the sites for several key enzymes including ion transporters such as the proton and calcium ATPases. The organization of microtubules and the synthesis of cell wall components are closely associated with the plasma membrane. However, technologies for the preservation of membrane structure and thereby extending the shelf life of fruits, vegetables, and flowers have become a reality only recently.

10.2 Phospholipase D and membrane deterioration

Phospholipase D is the key enzyme involved in the initiation of membrane deterioration. In response to hormones and external stimuli, phospholipase D is believed to become bound to the membrane initiating a cascade of catabolic reactions leading to the generation of several neutral lipids, the accumulation of which results in the destabilization of the membrane (Paliyath and Droillard, 1992). In general, enzymes such as phosphatidate phosphatise,

lipolytic acyl hydrolase, and lipoxygenase that act on intermediates generated during this catabolic cascade do not directly act on structural phospholipids. The activity of phospholipase D is also stimulated by calcium and low pH. Thus, if the action of phospholipase D is inhibited, then the rest of the enzymes are unable to act on the intermediates. This would prevent the accumulation of neutral lipids and the destabilization of the membranes. Theoretically, this should preserve or enhance the stability and function of the membrane, increasing the longevity of the produce.

As described in the previous chapter, phospholipase D is a complex enzyme in its structure and function. Association of phospholipase D to the membrane is believed to be driven by a hormone-stimulated increase in cytosolic calcium levels. If phospholipase D exists merely in the cytosol, it will not be able to catalyze the degradation of membrane phospholipids. The enzyme has two active sites and several cofactor-binding motifs that are believed to activate the enzyme. Thus, phospholipase D action may be inhibited by various strategies.

10.3 Phospholipase D inhibition by lysophosphatidylethanolamine

Inhibition of phospholipase D by lysophosphatidylethanolamine (LPE) was potentially the first PLD inhibition by a naturally occurring chemical agent that was documented and developed into a technology. Ryu et al. (1997) reported that LPE inhibited the senescence of fruits, flowers, and leaves. LPE also inhibited purified cabbage PLD in a dose-dependent manner. LPE was observed to be a noncompetitive inhibitor of PLD. The inhibitory activity was influenced by the length and unsaturation of acyl chains. The structural components of LPE, viz ethanolamine, and free fatty acids did not inhibit PLD activity, indicating the requirement for complete lysophosphatidyl ethanolamine structure for inhibitory activity. As well, other lysophospholipids such as lysophosphatidylcholine or lysophosphatidylglycerol did not inhibit PLD activity. A technology was developed for enhancing the shelf life and quality of fruits such as cranberry, tomato, and apple (Palta and Farag, 1992) through preharvest or postharvest application of LPE. This technology has been optimized for other commodities (www.nutrapark.com). LPE is also registered as a biopesticide (EPA factsheet 105120).

10.4 Phospholipase D inhibition by *N*-acylethanolamine

Some phospholipid head group such as choline has the property of inhibiting PLD action albeit at very high concentrations (>10 mM). Specific and effective inhibition of PLD (alpha isoform) by *N*-acylethanolamines (NAE) was reported by Austin-Brown and Chapman (2002). The ethanolamine moiety of phosphatidylethanolamine can be acylated to form *N*-acyl phosphatidylethanolamine, which is not a prevalent phospholipid in plants even though it has been claimed to occur naturally. PLD action on *N*-acylphosphatidylethanolamine would release *N*-acylethanolamine as the head group. *N*-acylethanolamines have been demonstrated to be potent inhibitors of PLD activity. NAEs with shorter chain length (C12) are more effective inhibitors than NAEs with a longer chain length (e.g., C18). As well, increasing the unsaturation of the acyl chain decreased the efficiency of inhibition. NAEs did not inhibit other PLD isoforms (beta and gamma) even though the catalytic domains of all PLDs show a high degree of similarity in sequences.

Technologies for the preservation of commodities including flowers, plant cuttings, trees, foliages, and fruits have been optimized (Chapman and Shea, 2007).

10.5 Phospholipase D inhibition by aliphatic aldehydes and alcohols

Phospholipase D has a unique mechanism of action. The phosphatidic acid–enzyme intermediate releases phosphatidic acid from the active site by hydrolysis. Instead of water, if the enzyme encounters a primary alcohol, then phosphatidic acid is coupled to the primary alcohol resulting in the formation of phosphatidyl alcohol. This reaction is referred to as transphosphatidylation. Thus, if plant tissues are treated with primary alcohols (methanol, ethanol, propanol, butanol, etc.), the resulting products vary from phosphatidylmethanol, phosphatidylethanol, and so on. Heating plant tissues in the presence of these alcohols enhances the reaction, and was the cause for artifact formation during phospholipid isolation. Incubating plant tissues in the presence of alcohols has also been used as an experimental technique to evaluate the activation of the enzyme in the presence of activators. However, such procedures are unreliable because of the compartmentalization of PLD and the inhibition of PLD activity by longer-chain primary alcohols.

While evaluating the effect of primary alcohols and aldehydes on phospholipase D activity in corn kernel fractions, we observed that primary alcohols and aldehydes can be effective inhibitors of PLD. A primary alcohol such as hexanol and an aldehyde such as hexanal were potent inhibitors of PLD activity (Paliyath et al., 2003). These components inhibited soluble as well as membrane-associated forms of PLD (Paliyath et al., 1999). It appears that as the chain length increases, the efficiency of primary alcohols in participating in the reaction decreases, essentially showing an inhibition of the reaction in the presence of water. Hexanal was a more efficient inhibitor of PLD activity. Aldehydes do not possess the primary alcohol structure required for their participation in the reaction and may block the reaction at the water-binding site. Hexanal is naturally produced during lipid peroxidation mediated by lipoxygenase and the hydroperoxide lyases and gives the characteristic green flavor evolved during wounding process in vegetables such as cucumber and beans. Hexanal is also a component of GRAS (generally regarded as safe) status.

The advantage of hexanal over other PLD inhibitors is its volatile nature. The produce can be exposed to a vapor if required, and this is similar to the application of 1-MCP (AgroFresh Inc.). We have also developed compositions containing hexanal along with ingredients such as geraniol, ascorbate, vitamin E, and calcium chloride. The composition for each produce may vary and has to be optimized. A basic stock compositions used for cherries include 1% (v/v) hexanal, 1% (v/v) geraniol, 1% (w/v) α-tocopherol, 1% (w/v) ascorbic acid, 0.1% (w/v) cinnamic acid, 0.1% (v/v) Tween 80, and 0.1% (w/v) $CaCl_2$, diluted in water to desired concentration (1–3% v/v). Calcium chloride may not be needed in some cases, for example, tomato. The compositions can be applied preharvest or postharvest. Usually, the preharvest application is conducted about 30 days and 15 days before harvest. In some fruits such as cherries, the formulation is applied 2 weeks and 1 week before harvest. The best effects for most fruits are observed at a dilution of 2% v/v in water. The stage of application is critical since phospholipase D inhibition has very little effect once the membrane deterioration is accelerated. For example, tomato fruits have to be treated at the mature green stage (just before the visible signs of yellow color) to obtain the best effects. Such treatments

Table 10.1 Effect of hexanal and its compositions on horticultural produce

Commodity	Treatment—preharvest spray	Treatment—postharvest spray or dip	Effects
Apple (Gala)	Hexanal formulation—2% v/v	Nil; air storage, 4°C	Delayed ripening, increased firmness, slightly reduced color
Apple (Delicious)	Hexanal compositions—1%, 2% v/v	Nil; air storage, 4°C	Increased firmness, reduced incidence of superficial scald
Apple (IdaRed)	Hexanal compositions—1%, 2% v/v		Reduced fruit drop
Apple (McIntosh)	Hexanal compositions—1%, 2% v/v	Nil; air storage, 4°C	Enhanced color, reduction in superficial scald
Apple (Cortland) (postharvest dip)	Nil	Hexanal composition—1% v/v; air storage, 4°C	Reduction in superficial scald
Banana (postharvest dip)	Nil	Hexanal compositions—1%, 2%, 3% v/v; air storage, 25°C	Enhanced shelf life, quality
Cherry (Bing)	Hexanal compositions—1%, 2% v/v	Nil; air storage, 4°C	Increased firmness, increased red color, delayed ripening, increased texture and sweetness
Cherry (Hedel)	Hexanal composition—1%	Nil; air storage, 4°C	Increased firmness, increased red color, increased texture and sweetness
Wine grapes, pinot noir, merlot, cabernet franc, chardonnay	Hexanal composition—1%	Nil	Increased brightness, reduced pathogen infection, increased juice yield
Table grape (Vanessa)	Hexanal composition—1%	Nil; air storage, 4°C	Increased brightness
Peach (Redhaven)	Hexanal composition—1%	Nil; air storage, 4°C	Increased firmness, increased red color, increased texture and sweetness
Pear (Anjou)	Hexanal composition—1%	Nil; air storage, 4°C	Increased firmness, increased texture and sweetness
Strawberry	Hexanal composition—1%	Nil; air storage, 4°C	Increased shelf life, enhanced quality and freshness
Strawberry	Nil	Hexanal vapor (0.01–0.05% w/w); air storage, 4°C	Increased shelf life, enhanced quality and freshness, reduced pathogens
Apples (fresh cut) (dip)	Nil	Hexanal composition—1%; air storage, 4°C	Increased shelf life, enhanced quality and freshness
Broccoli	Nil	Hexanal vapor (0.01–0.05% w/w); air storage, 4°C	Enhanced shelf life, reduced yellowing

(*continued*)

Table 10.1 (*Continued*)

Commodity	Treatment—preharvest spray	Treatment—postharvest spray or dip	Effects
Tomato (mature green stage, postharvest dip)	Nil	Hexanal compositions—1%, 2%, 3%; air storage, 12°C	Shelf life extended to nearly 2 months, increased firmness of ripe fruits
Fresh-cut vegetables (carrots, celery, cauliflower, cucumber, etc.)	Nil	Hexanal composition—1%; air storage, 4°C	Improved shelf life and quality
Carnations	Nil	Hexanal vapor (0.01–0.05% w/w); air storage, 25°C	Delayed senescence
Roses	Nil	Hexanal vapor (0.01–0.05% w/w); air storage, 25°C	Delayed senescence
Chrysanthemums	Nil	Hexanal vapor (0.01–0.05% w/w); air storage, 25°C	Delayed senescence

In the hexanal formulation, hexanal alone is the active ingredient. The hexanal composition, also referred to as an enhanced freshness formulation (EFF), also contains other ingredients (Paliyath and Murr, 2007). Treatments are provided as preharvest sprays, postharvest dips, or hexanal vapor treatments.

do not reduce the color or flavor development of tomatoes during further storage, but provide improved firmness. The effects of several trials on different produce are shown in Table 10.1.

Several aspects of this technology are under optimization. A combination of several active ingredients potentially provides beneficial effects by reducing deterioration by multiple mechanisms (Paliyath and Murr, 2007). For instance, a combination of PLD inhibition and the use of antioxidants would reduce phospholipid degradation as well as downstream oxidative processes. A total inhibition of PLD may be lethal, and a partial inhibition may provide added benefits. Inhibition of PLD activity by 30–40% through antisense PLD approach enhances several beneficial qualities (Chapter 9). Through the application of hexanal or its formulations, only a partial inhibition of PLD may be achieved. It is hypothesized that such an inhibition will reduce the requirements for carbon substrates to replenish the lost phospholipids through biosynthesis. This spared carbon sources then can be mobilized to the biosynthesis of beneficial quality, enhancing components through metabolite channeling as described in Chapter 21. The use of hexanal is also compatible for organic produce, as there are no postharvest treatment procedures available for organic produce currently. The PLD inhibition technology using hexanal is licensed to AgroFresh Inc., PA.

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Chapter 11

Heat Treatment for Enhancing Postharvest Quality

Susan Lurie

11.1 Introduction

In a time of increased awareness among consumers that many of the chemical treatments of fruits and vegetables to control insects, diseases, and physiological disorders are potentially harmful to humans, much effort is being directed into developing effective, nondamaging physical treatments for insect disinfestations and disease control in fresh horticultural produce. High- and low-temperature treatments, anoxia, and irradiation are some of the possibilities being employed. These treatments are generally applied commercially for insect and disease control. However, this chapter will discuss the additional effect of the high-temperature treatments on commodity physiology. High-temperature treatment of fruits or vegetables, whether it is applied for reasons of quarantine, decay control, or to affect product physiology, will have profound effects on the metabolism of the tissue. These effects include changes in tissue respiration, hormone production, particularly ethylene, enzyme activities, changes in confirmation of macromolecules including protein aggregation, in membrane components that can lead to increased membrane leakage, and other changes that can impact of fruit and vegetable quality (Lurie, 1998). Heat treatments include hot water dips, hot water brushing, and hot air treatments (vapor heat and forced air). All of these have to be adapted to the commodity being treated so as to achieve the desired effect, be it pest control, pathogen control, or direct effects on the commodity itself without causing damage. The type of high-temperature treatment and its duration will effect how it impacts on fruit or vegetable ripening or senescence as well as nutritional and quality attributes. This chapter will discuss some of the studies conducted in recent years to optimize procedures for high-temperature treatments and the effect of high temperature on commodity quality.

11.2 Effects on ripening

In climacteric fruits, which depend on ethylene for coordinated ripening, the high-temperature inhibition of ethylene can inhibit many ripening processes, including fruit softening, color changes, and aroma development. The synthesis of ethylene, which synchronizes the ripening processes of climacteric fruits such as tomatoes, is inhibited at temperatures near or above 35°C (Fig. 11.1). In apples heated in 38°C air,

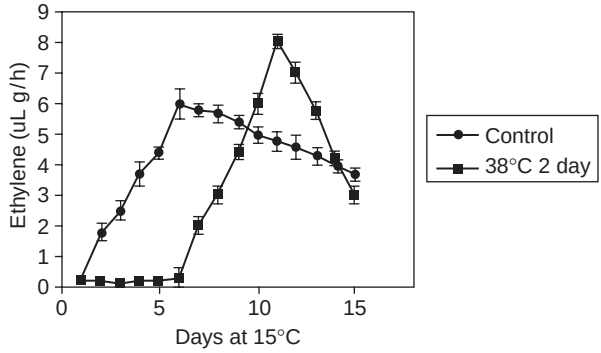

Fig. 11.1 The ethylene production from mature green tomatoes held at 15°C. Control tomatoes were placed directly in storage, and heated tomatoes were held for 2 days in 38°C air before storage. (Adapted from Lurie and Klein, 1992.)

1-aminocyclopropane-1-carboxylic acid (ACC) oxidase activity decreased 90% compared to untreated apples, which correlated well to the inhibition of ethylene produced by the heated apples, while ACC accumulated to higher levels in heated apples than in control apples (Klein, 1989). This indicated that a 38°C heat treatment inhibited ACC oxidase more than ACC synthase. However, ACC synthase was inhibited by a 38°C heat treatment, but more slowly than ACC oxidase in both apples (Roh et al., 1995) and kiwifruit (Antunes and Sfakiotakis, 2000). The inhibition of the ethylene pathway recovered slowly during apple storage after a heat treatment, and upon removal from storage ethylene production was often higher than from untreated fruit (Klein, 1989; Lurie and Klein, 1991). The heat-induced inhibition of ethylene synthesis was due both to direct inhibition of enzyme activity and to reduced synthesis of new enzyme. The abundance of mRNA of ACC oxidase was strongly depressed at 38°C (Lurie et al., 1996).

The inhibition of ripening due to lack of ethylene is reversible if the heat treatment is not too extended and does not cause damage. Volatile production inhibited by 38°C hot air treatment of apples, or 42°C hot water immersion of tomatoes recovered following the treatment, particularly if the fruit were stored for a period of time (Fallik et al., 1997; McDonald et al., 1998). Lycopene synthesis, inhibited by heat treatment of tomatoes, also recovered, and heat-treated tomatoes ripened normally (Lurie and Klein, 1992; McDonald et al., 1998). In fact, if the fruits were stored at low temperatures, the heat-treated tomatoes ripened normally (Fig. 11.2) while control fruits decayed as a result of chilling injury (Lurie and Klein, 1992). Polygalacturonase, an enzyme involved in digestion of cell walls leading to fruit softening is induced by ethylene. High temperature inhibited the activity of this enzyme and affected fruit softening in both mango (Ketsa et al., 1998) and tomatoes (Mitcham and McDonald, 1992). High temperature also inhibited β-mannanase and α- and β-galactosidase activities in tomatoes (Sozzi et al., 1997). Again the inhibition of these ripening processes of color development, volatile evolution, and softening, as with ethylene synthesis, was at the level of both enzyme activity and gene expression.

Nonclimacteric fruits also show effects of reduced softening rate and color development following heat stress. Strawberries, either hot air heated for 3 h or hot water heated for 15 min at temperatures from 40 to 50°C and then held at 20°C, had delayed color development and reduced firmness loss compared to unheated berries (Garcia et al., 1995; Civello

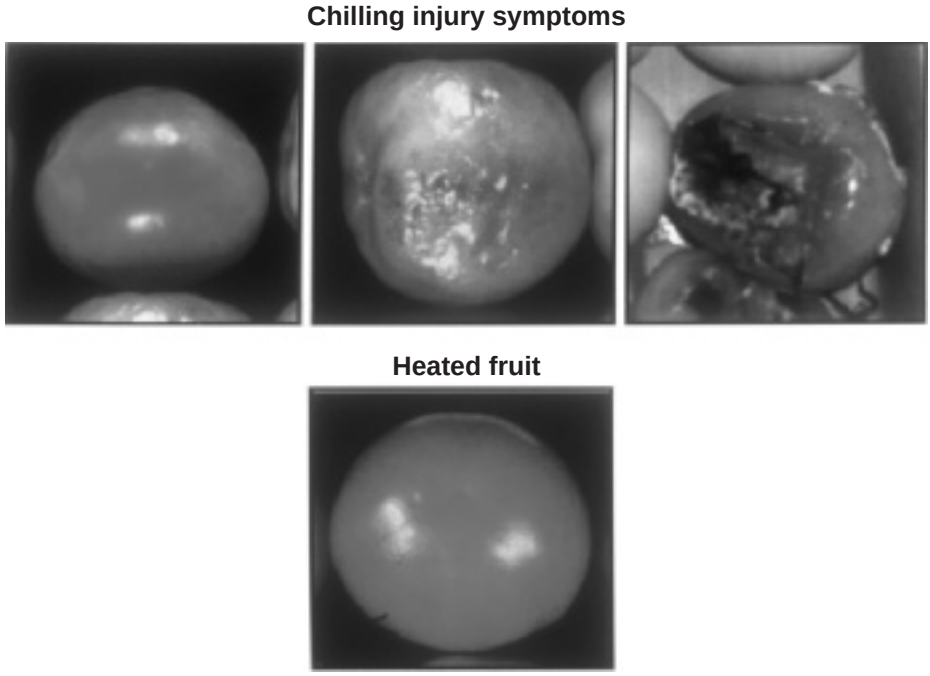

Fig. 11.2 Chilling injury symptoms in control fruit after storage at 2°C and normal ripening in fruit held at 38°C before 2°C storage.

et al., 1997). Therefore, the inhibition of ripening may also be a consequence of a general inhibition of gene expression at heat stress inducing temperature.

11.3 Effects on senescence

Broccoli senesces vary rapidly and the florets turn yellow after harvest as a result of chlorophyll degradation. Yellowing is one of the most important factors in quality degradation in this and many other green vegetables. Reproductive structures within the florets produce high amounts of ethylene, which play a role in broccoli senescence (Tian et al., 1994). High-temperature treatment with 45°C hot water for 10 min (Tian et al., 1996, 1997), 45°C for 14 min (Kazami et al., 1991a, b), 50°C for 2 min (Forney, 1995), or 50°C hot air for 2 h (Terai et al., 1999) delayed yellowing of harvested broccoli florets and reduced the rate of ethylene production. The different times and temperatures that were successful in this crop may be due to the fact that each study used different broccoli cultivars. The hot water treatments also slowed the loss of soluble proteins and ascorbic acid (Kazami et al., 1991b; Tian et al., 1997). The enzymes that promote chlorophyll degradation, chlorophyllase and peroxidase, were inhibited in the hot air-treated broccoli, while they increased in nonheated florets during storage (Funamoto et al., 2002, 2003) (Fig. 11.3).

In a similar manner, hot moist air at 40 or 45°C from 30 to 60 min reduced the senescence of harvested kale and collard leaves (Wang, 1998). Yellowing of the leaves was delayed, losses of sugars and organic acid were slower, and turgidity was maintained during storage

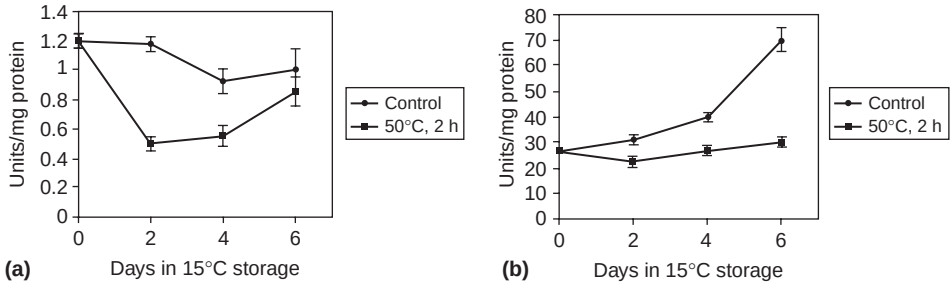

Fig. 11.3 Chlorophyllase (a) and chlorophyll degrading peroxide, and heated florets were held in hot air at 50°C for 2 h before storage. (Adapted from Funamoto et al., 2002, 2003.)

after the heat treatments. However, the same treatments applied to Brussels sprouts were not effective in delaying yellowing.

Other processes that involve ethylene in harvested vegetables, such as geotropic bending of asparagus (Paull and Chen, 1999) and extension growth of green onions (Hong et al., 2000; Cantwell et al., 2001), were also controlled by hot water treatments before storage. Hot water dips controlled sprout and root growth in garlic cloves (Cantwell et al., 2003). Sprouting and spoilage of potatoes were also inhibited by a hot water dip (Ranganna et al., 1998). The best treatments to inhibit these physiological processes were hot water dips from 2 to 4 min in the temperature range of 50–55°C.

11.4 Effects on chilling injury

The storage temperatures of subtropical fruits and vegetables are arrived at by a compromise between temperatures, which are low enough to inhibit ripening processes, but cause chilling injury, and those which are high enough to avoid chilling injury, but do not prevent the continuation of ripening. Inducing resistance in a fruit or vegetable to chilling injury will allow it to be stored at a lower temperature. This in turn may allow transport in ships rather than the more costly airfreight. In addition, a preshipping heat treatment can allow for low-temperature disinfestations of commodities, such as citrus, by improving the resistance of fruit to the chilling injury generally incurred during this treatment. Table 11.1 tabulates some of the commodities that have been tested for high-temperature induction of resistance to low-temperature injury. The table is partial because this is a dynamic field of research and new studies are appearing regularly.

What is apparent from the table is that a time–temperature regime can be found for any commodity that will reduce chilling injury. The successful treatment is arrived at empirically, by trial and error, and what works on one cultivar may not be as successful on another. For example, Israeli citrus fruit respond well to a hot water dip of 3 min at 53°C (Rodov et al., 1995). A number of citrus varieties including grapefruit, lemon, oroblanco, and kumquat all had reduced chilling injury after these two treatments and low-temperature storage. However, "Tarocco" blood oranges grown in Italy will be heat damaged by a hot water dip of 3 min at 53°C, but respond favorably to 3 min at 50°C by reduced chilling injury (Schirra et al., 1997). Another example of differences among cultivars involves hot air treatment. A hot air prestorage treatment of 2 days at 37°C caused off-flavors to develop in "Tarocco" blood oranges (Schirra et al., 2002), but was helpful in controlling chilling

Table 11.1 Physiological benefit of thermal treatments to prevent chilling injury in horticultural crops

Crop	Phenomenon/appearance	Regime	Temperature/time
Apple	Scald	HAT[a]	38°C/4 days or 42°C/2 days
Avocado	Skin browning	HAT then HWT	38°/3–10 h then 40°/30 min
	Internal browning, pitting	HWT	38°/60 min
Cactus pear	Rind pitting, brown staining	HAT or HWT	38°/24 h or 55°/5 min
Citrus	Rind pitting	HAT	34–36°/48–72 h
		HWT	50–54°/3 min; 53°/2–3 min
		HWB	59–62°/15–30 s
Mango	Pitting	HAT	38°/2 days; 54°/20 min
Persimmon	Gel formation	HWT	47°/90–120 min;
		HAT	50°/30–45 min; 52°/20–30 min
Green pepper	Pitting	HAT	40°/20 h
Cucumber	Pitting	HWT	42°/30 min
Tomato	Pitting	HAT	38°/2–3 days
		HWT	48°/2 min; 42°/60 min
Zucchini	Pitting	HWT	42°/30 min

[a]HAT, hot forced-air treatment; HWB, hot spray and brush treatment; HWT, hot water treatment.

injury in "Fortune" mandarins (Martinez-Tellez and Lafuente, 1997), and reduced decay as well as chilling injury in lemons (Rodov et al., 1995).

Both hot water and hot air treatments control chilling injury on a number of fruits. Citrus has already been mentioned earlier. However, avocados, mangos, and persimmons can also benefit from either hot air or hot water. Chilling injury was prevented in avocados by short hot air treatments at 38°C (up to 10 h), or by 30 min in hot water from 39 to 42°C (Nishijima et al., 1995; Florissen et al., 1996; Hofman et al., 2002). Persimmons, under similar hot air or hot water treatments as avocados, also had less chilling injury (Burmeister et al., 1997; Lay-Yee et al., 1997; Woolf et al., 1997a, b). Persimmons are an interesting case in which the chilling-injury symptoms include a change in fruit texture to mealiness or gel, which is related to changes in the pectin polysaccharide component of the cell walls of the fruit. Two enzymes of pectin, pectin esterase and polygalacturonase, when they work in concert, metabolize pectin to smaller moieties and lead to fruit softening. When polygalacturonase is inhibited, but pectin esterase is still active, pectin polysaccharides can swell into a gel-like structure. The high-temperature treatment of persimmons prevents low-temperature inactivation of polygalacturonase and prevents the deleterious fruit texture changes.

Superficial scald is a low-temperature physiological disorder of certain apple and pear cultivars that develops during prolonged low-temperature storage. Typically, early-harvested and less mature fruits are most susceptible, but scald may also develop on fully mature fruit. The disorder appears as browning of the skin as a result of damage to the hypodermal cells. Scald development results from production of α-farnesene and its auto-oxidation to conjugated trienols. Generally, correlations between conjugated triene concentration and scald occurrence are strong, but those between α-farnesene and scald are variable (Meir and Bramlage, 1988). α-Farnesene typically increases rapidly during storage and then declines, while conjugated trienes continue to increase (Watkins et al., 1995).

Prestorage heat treatments have been found to be effective in controlling scald for the first 4 months of regular air storage, and for much longer if the fruits are held in controlled atmosphere storage (Lurie et al., 1990). Hot air treatment has been effective in controlling

Fig. 11.4 Superficial scald on apple peel is prevented by diphenylamine (DPA) and by hot air heat of 12 h at 46°C or 72 h at 38°C.

the disorder, and a number of time–temperature regimes have been effective, from 3 days at 38°C to 12 h at 46°C (Klein and Lurie, 1992) (Fig. 11.4). Recently there have been reports of some success with a hot water dip at 48°C (Jemric et al., 2006). Heat treatments that satisfy quarantine requirements tried on apples also controlled scald, but exacerbated another disorder, bitter pit (Neven et al., 2000). In addition, trials on a number of cultivars showed that their response to high temperature differed, with some responding positively and others developing internal browning (Tu and De Baerdemaeker, 1997). Therefore, it is important to test the cultivar at different heating regimes and not simply to use what has been reported for other cultivars.

11.5 Heat treatment in fresh-cut fruits

The use of heat treatment on fresh-cut commodities can be a treatment of the commodity before processing or a treatment after processing. In the first instance, the purpose would be to affect ripening or senescence processes so that these processes will be delayed in the fresh-cut commodity. The reduced shelf life of cut fruit, relative to that of intact fruit, is associated with physiological and biochemical changes typical of the senescence process such as increased respiration and ethylene production, and loss of membrane integrity (Toivonen and DeEll, 2002). The inhibition that a heat treatment confers on whole fruit can extend to the fruit after cutting. This has been tested in a number of fruits. In melons a precut heat treatment at 50°C for 60 min reduced the rate of respiration and moisture loss of the cut fruit, and increased the intensities of fruity and sweet aromatic flavors (Lamikanra et al., 2005). Apples were also heat treated before slicing. When they were held for 4 days

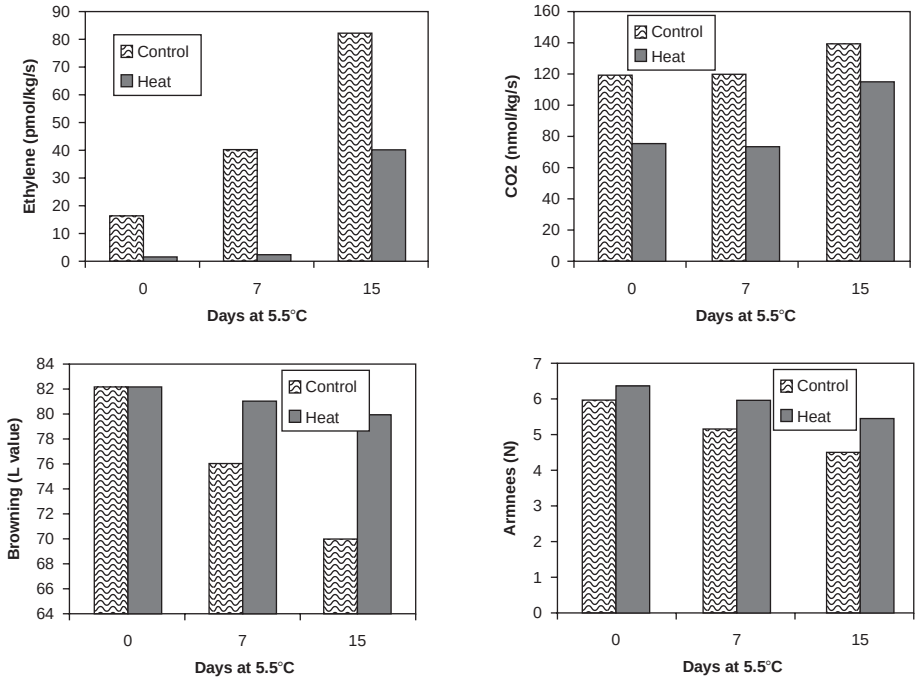

Fig. 11.5 The effect of heating apples in hot air for 4 days at 38°C before slicing on ethylene production, respiration, slice browning, and firmness of the slices held at 5.5°C, compared to unheated sliced control apples. (Adapted from Bai et al., 2004.)

at 38°C before slicing respiration, ethylene production and softening were reduced, but acidity loss was high and aroma volatiles were lower (Bai et al., 2004). The slices from heated apples also showed less browning (Fig. 11.5). Shelf life at 5.5°C was 50% longer than for control apples. In another study, apples were held at 34–42°C for less than 70 min before slicing. The treatments prevented cut-surface browning and softening during storage at 5°C (Barrancos et al., 2003). A similar study was performed with "Rocha" pears (Abreu et al., 2003). Heating the fruit from 35 to 45°C for 40–150 min before processing avoided cut-surface browning and maintained firmness, pH, and soluble solids content (SSC) during storage at 2°C. Two studies on fresh-cut fruit have looked at combining heat with a $CaCl_2$ dip to maintain firmness during storage. In both fresh-cut cantaloupe and mangoes, a dip in hot water with 2.5–3.5% $CaCl_2$ improved firmness of the fruit during storage at 5°C (Luna-Guzman et al., 1999; Trindale et al., 2003). Dipping in hot water was more effective than ambient water with $CaCl_2$.

One of the largest commodities marketed as fresh-cut is lettuce. Many studies have looked at the effect of heat treatments on lettuce from various aspects, including reduction of plant and human pathogens, and inhibition of senescence processes. Cut iceberg lettuce washed in chlorinated water at 50°C found that high temperature reduced the microbial populations more than at 4°C (Delaquis et al., 2004). The treatment also delayed the appearance of tissue browning (Fig. 11.6). A 50°C treatment with or without chlorine treatment reduced aerobic mesophiles, psychrotrophs, Enterobacteriaceae, molds, and lactic acid bacteria, and also delayed tissue browning (Li et al., 2001b). A study was performed

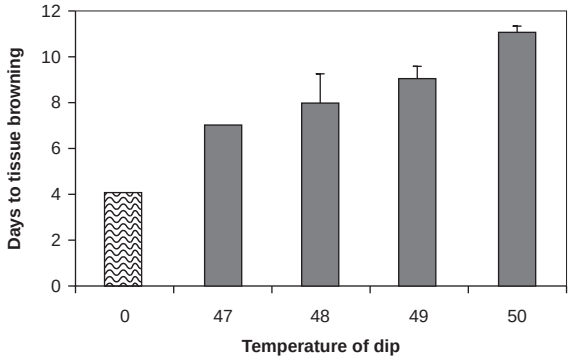

Fig. 11.6 Cut iceberg lettuce dipped in chlorinated water at 4°C or in 47–50°C water for 20 s and then held at 4°C. The high-temperature-treated lettuce took longer to develop tissue browning. (Adapted from Delaquis et al., 2004.)

using this 50°C dip with 20 ppm chlorine on *Escherichia coli* O157:H7 inoculated onto fresh-cut iceberg lettuce. It was found that the pathogen population decreased when lettuce was held at 5°C and increased when held at 15°C without respect to a chlorine dip (Li et al., 2001a). Another study looked at *E. coli* O157:H7 and *Salmonella enterica* and how a dip in alkaline-electrolyzed water at 50, 20, or 4°C affected pathogen populations (Koseki et al., 2004). A treatment of 50°C for 1 or 5 min resulted in 2–4 log 10 cfu/g reduction in both pathogens and showed no deleterious effects on the lettuce. However, in this study no storage was performed. Other methods of sanitizing lettuce and preventing the growth of human pathogens include the use of lactic acid or hydrogen peroxide combined with hot water. A trial against *E. coli* O157:H7, *S. enterica*, and *Listeria monocytogenes* looked at both lactic acid and hydrogen peroxide and found that the best treatment for reduction of the pathogens and maintenance of sensory quality of the lettuce was 2% hydrogen peroxide at 50°C for 60 s (Lin et al., 2002). When this treatment was tested in sensory trials within supermarkets, the preferred lettuce was after antibacterial treatment (McWatters et al., 2002).

Wounding caused by making fresh-cut lettuce causes accumulation of phenolic compounds, which cause tissue browning, and treatments of 2.5 min heat at 45°C or 90 s at 50°C will reduce this accumulation. It does this by preventing the increase in phenylalanine ammonia lyase activity that accompanies wounding, and also by inducing the synthesis of heat shock proteins (Saltveit, 2000; Loaiza-Velarde and Saltveit, 2001).

11.6 External damage

Damage can appear as peel browning (Kerbel et al., 1987; Klein and Lurie, 1992; Lay-Yee and Rose, 1994; Schirra and D'hallewin, 1997), pitting (Jacobi and Gowanlock, 1995), or yellowing of green vegetables such as zucchini (Jacobi et al., 1996) or cucumber (Chan and Linse, 1989). One of the most common types of damage observed following a heat treatment is surface scalding. "Manila" mangoes showed severe skin scalding when forced-air heated at temperatures of 45°C or higher, slight skin scalding from heating at 44°C and no damage at 43°C, indicating the presence of a threshold temperature for skin injury to develop

Table 11.2 Response of fruits to various exposure times and temperatures with respect to phytotoxic effects and aspects of ripening such as softening, skin color changes, sugar, and acidity

Fruit	Method[a]	Time	Temperature (°C)	Phytotoxic symptoms	Softening	Color
Apples	HA	96 h	38	N	Firmer	Increased
	HA	96 h	43	Y	Firmer	Increased
Oroblanco citrus	HA	4 h	46	N	Softer	Increased
	HA	4 h	47	Y	Softer	Increased
Mango	VH	15 min	46	N	No effect	No effect
	VH	30 min	46	N	Softer	Increased
	VH	30 min	47	Y	Softer	Increased

Lurie (unpublished).
[a]HA, hot air; VH, vapor heat.

(Ortega-Zaleta and Yahia, 2000). This is true of other fruits as well (Table 11.2). Hot water treatments can also result in damage to the epidermis of the commodity. Red ginger flowers heated in water at 49°C for 12–15 min had some damage to the inner bracts that resulted in necrotic tissue (Hara et al., 1996). The authors also found that hot water treatment caused an intensification of mechanical injury in flowers, emphasizing the importance of careful handling of materials destined for heat treatment. Surface browning of peaches exposed to hot water treatments increased with both time (1.5–5 min) and temperature (50–55°C) (Phillips and Austin, 1982). Fruit differed in susceptibility because of seasonal and maturity effects.

Many researchers have shown that heating in moist forced air was less damaging to the fruit than heating in hot water or vapor-heated air. Shellie and Mangan (2000) closely monitored fruit surface temperatures during heating with hot water, moist forced air, and water vapor-saturated air (vapor forced air) (Fig. 11.7). The temperature of the fruit surface varied according to the heating medium used. Fruit surface temperature was coolest when heated with moist forced air and reached only 81% of the temperature of the heating medium after 5 min of exposure. The surface of the fruit heated with vapor-saturated air or hot water reached 96% of the temperature of the heating medium within 5 min. During heating with vapor-saturated air, the surface temperature of the fruit exceeded the air temperature after about 20 min of heating and remained 1°C higher, while the surface temperature of fruit

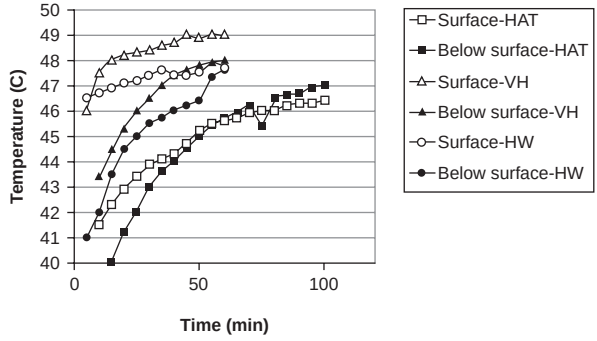

Fig. 11.7 Rate of heating of fruit at surface of 2 mm below the surface when heated in 48°C moist, forced-air treatment (HAT), vapor-saturated, forced air (VH), or hot water (HW).

heated in moist forced air remained cooler than the air. The authors suggest that the latent heat of condensation may be responsible for the hotter surface temperature during vapor forced-air heating, and that evaporative cooling may result in the lower surface temperatures in moist forced air. These data indicate that the water vapor pressure during forced-air heating influences the surface heat transfer coefficient (Shellie and Mangan, 2000) and has a tremendous influence on the potential to develop skin scalding.

11.7 Internal damage

Internal injury to fruit can also occur as a result of heat treatment, sometimes in the absence of any external damage. Internal damage can include flesh darkening in avocado, citrus, lychee, nectarine, and sapote mammey (Jacobi et al., 1993; Shellie et al., 1993; Lay-Yee and Rose, 1994; Diaz-Perez et al., 2001; Follett and Sanxter, 2003). Internal damage can appear in mango and papaya as poor color development, abnormal softening, the lack of starch breakdown, and the development of internal cavities (An and Paull, 1990; Mitcham and McDonald, 1993). Jacobi et al. (1996) observed injuries to mango fruit from hot water treatment, which included surface damage but also internal cavities and starchy layers beneath the skin (Fig. 11.8). Traditional heat treatments naturally contain a controlled atmosphere effect because of the modification of internal atmospheres during treatment. Mitcham and McDonald (1993) demonstrated that CO_2 increased to 13% and O_2 decreased to 6% in mangoes subjected to high-temperature forced-air treatments and was related to the development of internal cavitation in the mango fruit. Esquerra and Lizada (1990) showed a similar modification of the internal atmosphere of “Carabao” mangoes following vapor heat treatment. Vapor heat-treated mangoes increased in internal cavitation from a range of

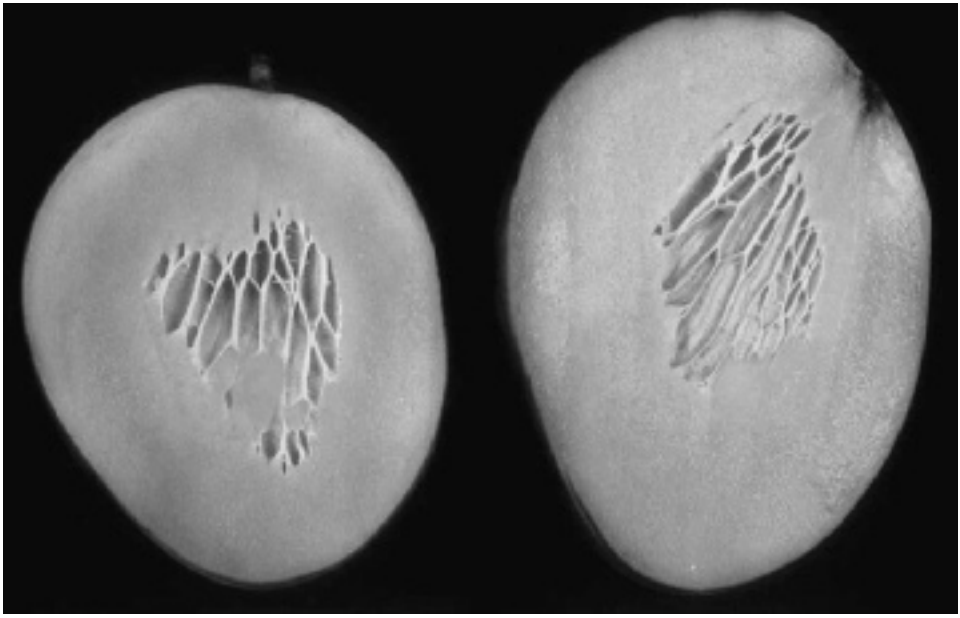

Fig. 11.8 Internal injury in mango fruit caused by hot air treatment.

0–17% to 66–75% when heated to 46°C for 1½ h (Esquerra and Lizada, 1990). Internal cavitation has also been observed in "Hayward" kiwifruit heated at 40°C in controlled atmosphere (0.4% O_2 + 20% CO_2) for 10 h when the fruits were not hydrocooled after treatment (Lay-Yee and Whiting, 1996). Internal cavitation can also develop in mango fruit from exposure to high temperatures in the field (Gunjate et al., 1982) or from exposure to modified atmospheres, especially at ambient temperatures in the tropics (Gautam and Lizada, 1984).

11.8 Conclusions

Application of heat treatments to plant materials as a means of controlling pests or pathogens provides a nonchemical method of control. However, the tolerance to such treatments must be carefully evaluated. Heat treatment can have beneficial effects beyond pest control such as reducing susceptibility to chilling injury and reducing the rate of ripening. Heat damage may be immediate or may develop after a period of storage. Tolerance to heat exposure is influenced by species, cultivar, harvest maturity, growing conditions, and handling between harvest and treatment. In addition, the method used to apply heat can greatly influence product tolerance. In some cases, special treatments can be applied to increase product tolerance to a heat treatment, but one must also consider if this will influence treatment efficacy against insect pests or pathogens.

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Chapter 12

The Role of Polyphenols in Quality, Postharvest Handling, and Processing of Fruits

H.P. Vasantha Rupasinghe

12.1 Introduction

Phenolics are a chemically heterogeneous group of over 12,000 plant secondary metabolites that are ubiquitous in plants. A phenolic is a chemical compound characterized by at least one aromatic ring (C_6) bearing one or more hydroxyl group. Some of them contain carboxylic acids and glycosides and are water soluble. Some are insoluble in water because of complex structure. In general, the principal ecological functions of plant polyphenols are to adapt the plant to changing biotic and abiotic environment; to defend plants against herbivores and pathogen attacks; and to act as signal molecules to interact with the environment.

Pigments such as many colorful polyphenols, especially anthocyanins, serve as attractants for fruit- or seed-dispersing animals. Among pigments, a wide range of anthocyanins, other flavonoids, and carotenoids have been characterized in fruits. Plant pigments also provide protection against environmental stress preventing cellular damage from free radicals generated due to stressful conditions. For example, some phenolic compounds can absorb light at shorter wavelengths and therefore can protect plants from ultraviolet (UV) irradiation. Since polyphenols possess strong antioxidant properties, they also play a role in mitigating the oxidative stress during postharvest storage and handling, and thus contribute to extending the shelf life of fruits. Polyphenols such as lignins contribute to the firmness of fruits, protecting the fruits against mechanical injuries during postharvest handling. Polyphenols present in fruit skin is implicated as a factor, which contributes toward the resistance of some apple cultivars to the postharvest physiological disorder, superficial scald.

Pigments are important feature of fruits both from the ecological perspectives as well as from human use, since pigments are indicators of fruit maturity and ripeness. Polyphenols are also among the major determinant factors for the quality, that is, color, aroma, bitterness, and astringency of fresh fruits as well as fruit products such as juices, beverages, wines, jellies, and jams. Polyphenols such as tannins interact with proteins and polysaccharides of processed food products and sometimes form hazes or precipitates and also impact on the nutritional value. The taste (bitterness) and astringency of fruits and their products are

mainly influenced by many polyphenols especially oligomeric proanthocyanidins, certain hydroxycinnamic derivatives, and flavanones.

Recently, phenolics present in fruits and vegetables have attracted increased attention in the field of nutrition, health, and medicine largely because of their antioxidant properties and potential health benefits (Graf et al., 2005). As a result, during the last decade, the consumer demand for fruits has been increasing significantly due to the understanding of health-promoting properties of polyphenols and other phytochemicals present fruits. Most importantly, value-added fruits such as fresh-cut fruits have been introduced to the market, and the demand for such products is steadily increasing. Conversely, the fresh-cut produce industry is challenged with postcut enzymatic browning of many fruits due to the oxidation of specific polyphenolic compounds by polyphenol oxidase (PPO).

Also, fruit phenolic compounds have applications in the food industry as natural colorants, fragrants, antioxidants, and antimicrobial agents (Cowan, 1999; Muthuswamy and Rupasinghe, 2007). There is an increasing demand for natural products such as polyphenols for replacing the synthetic food additives that are implicated with possible toxic effects at certain concentrations. Polyphenols are also considered as food supplements or value-added food ingredients (Rozek et al., 2007). Recently, the potential to incorporate polyphenolics extracted from fruit and vegetable wastes in cosmetic products has also been explored (Peschel et al., 2006).

In this chapter, an overview is provided on the nature of polyphenols in fruits, their biosynthesis and regulation, and selected roles of polyphenols in postharvest and processing. The information provided in this chapter will give an insight to the postharvest biologists and food processors working on different aspects of fruit polyphenols.

12.2 Structural diversity and classification of plant polyphenols

Plant phenolic compounds can be classified into different classes according to the nature and the number of carbon atoms of their carbon skeleton (Table 12.1). The structure of natural polyphenols varies from simple molecules, such as phenolic acids, to highly polymerized compounds, such as condensed tannins (Harborne, 1980). In general, phenolic biosynthesis occurs through several different routes, and therefore, produces a heterogeneous group of compounds. However, two basic pathways are involved: the shikimic acid pathway and the malonic acid pathway. Most of the plant phenolics are derived from the shikimic acid pathway. In contrast, malonic acid pathway is less significant in the biosynthesis of polyphenols in higher plants, but is predominant in fungi and bacteria. The shikimic acid pathway converts simple carbohydrate precursors derived from glycolysis and the pentose phosphate pathway to the aromatic amino acids. One of the pathway intermediates is shikimic acid, which has given its name to this whole sequence of reactions. The shikimic acid pathway is present in plants, fungi, and bacteria but is not found in animals. Animals are not able to synthesize the three aromatic amino acids: phenylalanine, tyrosine, and tryptophan, which are therefore essential nutrients in animal diets. Although plant phenolics exist in a large variety, most of them are typically derived from phenylalanine and share the common C_6–C_3 carbon backbone of the phenylpropanoid unit. By incorporating one or more hydroxyl group(s) into the phenyl ring, different phenolics are formed.

Table 12.1 The major classes of phenolic compounds present in fruits

Number of carbons	Carbon skeleton	Class and subclass	Example
6	C_6	Simple phenols	Catechol
7	C_6—C_1	Hydroxybenzoates (phenolic acids)	Gallic acid, protocatechuic acid, syringic acid, gentisic acid, *p*-hydroxybenzoic acid
9	C_6—C_3	Hydroxycinnamates (phenolic acids)	Caffeic, *p*-coumaric, ferulic, isoferulic
		Coumarins	Scopoletin, aesculetin, umbelliferone, limmettin, herniarin
10	C_6—C_4	Naphthoquinones	Juglone
13	C_6—C_1—C_6	Xanthones	Mangiferin, mangostin, gartanins
14	C_6—C_2—C_6	Stilbenes	Resveratrol
15	C_6—C_3—C_6	Flavonoids	
		Flavanone	Naringenin, hesperetin, neohesperidin, eriodictyol, citromitin, pinostrobin
		Flavone	Luteolin, apigenin, tangeretin, nobiletin, sinensetin
		Flavonol	Quercetin, kaempferol, myricetin
		Flavan-3-ol	Catechin, epicatechin, gallocatechins, epigallocatechin, epicatechin gallate
		Anthocyanins	Cyanidin, delphinidin, malvidin, peonidin, petunidin, pelargonidin
		Dihydrochalcones	Phloretin, phloridzin
18	$(C_6$—$C_3)2$	Lignans	Secoisolariciresinol, matairesinol
30	$(C_6$—C_3—$C_6)2$	Biflavonoids	Amentoflavone
n	$(C_6$—$C_3)n$	Lignins	Guaiacyl lignins
n	$(C_6$—$C_1)n$: Glu	Hydrolysable tannins	Gallotannins, ellagitannins, castalagin, corilagin, chebulagic acid
n	$(C_6$—C_3—$C_6)n$	Condensed tannins (flavolans)	Procyanidins (catechin polymers)

Adapted from Harborne (1980).

12.3 Distribution of phenolic acids in fruits

In literature, both hydroxybezoates and hydroxycinnamates and their derivatives are referred collectively as phenolic acids. Phenolic acids are found predominantly and widely in almost all fruits (Herrmann, 1989) (Table 12.2). The most commonly found hydroxycinnamic acids are *p*-coumaric, caffeic, ferulic, and sinapic acids, while *p*-hydroxybenzoic, protocatechuic, vanillic, and syringic acids are the common hydroxybenzoic acids in fruits. These derivatives differ in their structure only by the degree of the hydroxylations and methoxylations of their aromatic rings. In fruits, phenolic acids are commonly found as conjugated forms with quinic acid. It seems that chlorogenic acid, which is derived from caffeic and quinic acids, is the most common hydroxybenzoic acid derivative in berry, pome, and stone fruits (Mattila et al., 2006). The general term "chlorogenic acid" includes three positional isoforms. These isoforms differ from one another based on which hydroxyl group of quinic acid is used to combine the two precursor molecules, quinic acid and caffeic acid. The following common (and chemical) names have been given to these isoforms: chlorogenic acid (5-caffeoylquinic acid), cryptochlorogenic acid (4-caffeoylquinic acid), and neochlorogenic acid (3-caffeoylquinic acid). The presence of chlorogenic acid

Table 12.2 Concentrations (mg/100 g fresh weight) of phenolics of selected fruit crops

Fruit	Total phenols	Hydroxycinnamic acid derivatives	Anthocyanins	Flavonols	Flavan-3-ols	Tannins
Apples	50–1,100	6–134	10–2,160 (P[a])	18–41 (P)	0.2–16	400–3,500
Blueberry	381–4,651	188–211	160–503	2.4–2.9	5–20	
Cranberry			46–172	14–33	6–20	100
Grape (white)	350 (P)	1.3–87 (P)	0	8.1–8.2	1.4–53 (P)	17 (P)
Grape (red)	900–950	10–109 (P)	8.388	1.9–10	220–370	32–78 (P)
Orange	831	14–16	050–100			
Peach	28–180	8–75	0	0.4–1	5.3–14	90–120
Pear	123–400	170	5–10 (P)	4–160 (P)	0.1–6.2	53
Plum	167–200	12–94	2–5.3	2–5.2	1.8–6.1	76–150
Strawberry	85	1.4–3.1	28–70	2.1–17	2.4–9.6	110–150

Adapted from Kalt (2001).
[a] "P" indicates the peel tissue.

and other hydroxycinnamates in fruits and fruit products especially wine and cider are primarily responsible for bitterness and astringency (Marvin and Nagel, 1982).

Chlorogenic acids possess various antioxidant properties. In an in vitro study by Meyer et al. (1998), chlorogenic has been shown to inhibit the oxidation of low-density lipoprotein (LDL) by 86–91% at a concentration of 5 μM. The prevention of LDL oxidation has been associated with decreased incidence of atherogenesis and coronary heart disease. Chlorogenic acid has also been attributed to having inhibitory effects on glucose-6-phosphate translocase (Gl-6-P translocase). This in turn can reduce the hepatic glucose production and the severity of non-insulin-dependent diabetes mellitus (Hemmerle et al., 1997). Anticarcinogenic activity has also been shown to exist in chlorogenic acid. Chan et al. (1986) have demonstrated that chlorogenic acid has the ability to suppress the mutagenic properties of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) in *Salmonella typhimurium* strain TA1535. By feeding hamsters a diet composed of 0.025% chlorogenic acid for 24 weeks, the incidence of methylazoxymethanol acetate-induced liver and bowel cancer was reduced (Mori et al., 1986).

12.4 Distribution of flavonoids in fruits

Flavonoids represent the most common and widely distributed group of phenolics in fruits. More than 6,000 different flavonoids have been characterized from higher plants. Their common structure is C_6–C_3–C_6 and consists of two aromatic rings linked through three carbons that usually form an oxygenated heterocycle (Harborne, 1980). Figure 12.1 shows the basic structure and the system used for the carbon numbering of the flavonoid nucleus. Structural variations within the rings subdivide the flavonoids into several families: flavanone, flavonols, flavones, flavanols, anthocyanidins, dihydrochalcones, and others. These flavonoids often occur as glycosides, glycosylation rendering the molecule more water soluble and less reactive toward free radicals. Further modification occurs at various stages, resulting in an alteration in the extent of hydroxylation, methylation, isoprenylation, dimerization, and glycosylation (producing O- or C-glycosides). The sugar most commonly involved in glycoside formation is glucose, although galactose, rhamnose, xylose, and arabinose also occur, as well as disaccharides such as rutinose. Phenolic compounds act as

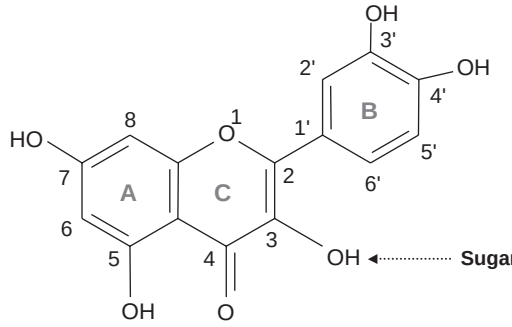


Fig. 12.1 Chemical structure of quercetin (a flavonol) to demonstrate the labeling of aromatic rings and numbering of the structure. The in vitro antioxidant properties of quercetin compounds are suggested depending on three structural characteristics: 3′4′-dihydroxy phenyl groups of B ring; 2,3 double bond and 4-carboxy of C ring; and 3-hydroxy substitution/glycosylation. Sugar substitution at C-3 position is common for naturally occurring quercetins of fruits.

antioxidants with mechanisms involving both free radical scavenging and metal chelation. They have ideal structural chemistry for free radical–scavenging activities, and have been shown to be more effective antioxidants in vitro than vitamins E and C on a molar basis (Rice-Evans et al., 1997).

In term of their role in fruits, flavonoids help to protect the fruit against damage by UV light. The phenolic compounds also play an important role in fruit ripening by giving the desired color and flavor to the fruit as in cherries, apples, plums, grapes, etc., and attracting humans, animals, birds, and insects for seed dispersal. Besides, these polyphenolics serve as parameters for judging the quality of the fruit and fruit products that include color, flavor, texture, and shelf life. When fruit is unripe, the content and type of phenolic compounds is different from the ripe fruit. For example, unripe fruits usually have high tannin content that help to protect the fruit from environmental factors and to prevent from herbivores animals. The phenolic compounds contributing to the flavor include tannins (hydrolysable and condensed), which are associated with astringency in ciders, wine, and semidried banana products. Citrus fruits such as grapefruits contain naringin as predominant bitter flavanones, whereas, in oranges, naringin and neohesperidin are responsible for the bitterness (Rouseff et al., 1987). Hence, the presence of the phenolic compounds is a major criterion for the consumer selection of these fruits.

Recently, flavonoids have attracted a growing interest for their potential health benefits in human health and nutrition, and the mitigation of numerous chronic diseases including various types of cancers, cardiovascular diseases, and neurodegenerative disorders (Boyer and Liu, 2004).

12.5 Biosynthesis of polyphenols

The major polyphenolics present in fruits belong to subgroups of phenolic acids (hydroxybenzoates and hydroxycinnamates) and flavonoids. Precursor of the majority of fruit phenolic compounds is the aromatic amino acid, L-phenylalanine. The biosynthesis of phenylalanine occurs through the shikimate or arogenate pathway (Fig. 12.2). The shikimate pathway begins with the condensation of erythrose 4-phosphate (an intermediate of the pentose phosphate pathway) and phosphoenolpyruvate (PEP, a glycolytic intermediate) to

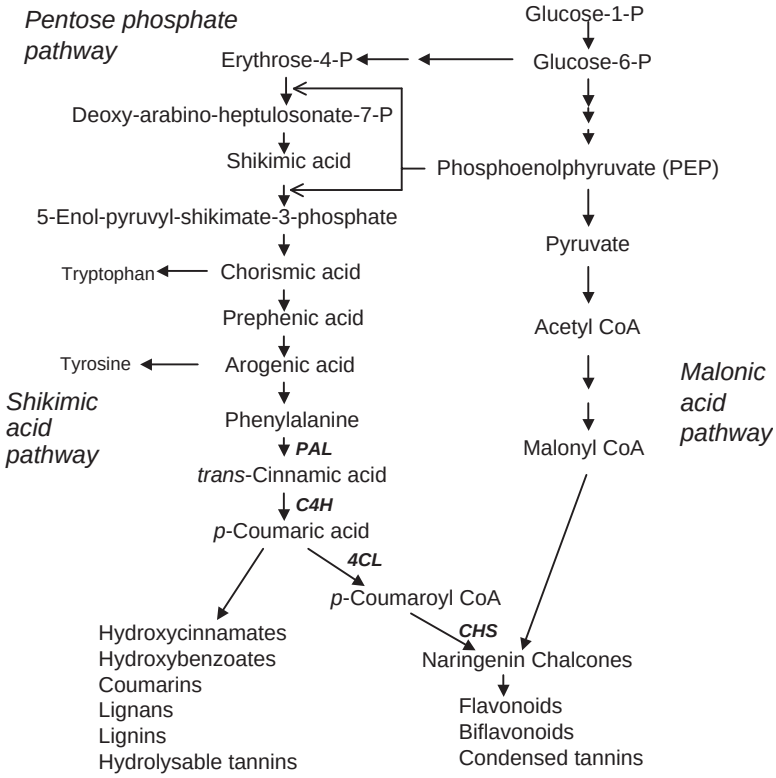


Fig. 12.2 Biosynthetic pathways of major polyphenolic groups present in fruits. Indicated enzymes are as follows: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-hydroxycinnamoyl CoA ligase; CHS, chalcone synthase.

produce deoxy-arabino-heptulosonate-7-phosphate, which then converts to shikimic acid (shikimate). Condensation of shikimic acid with another molecule of PEP results in the formation of 5-enol-pyruvyl-shikimate-3-phosphate (EPSP). Conversion of EPSP to chorismic acid (chorismate) is catalyzed by chorismate synthase by elimination of phosphate from EPSP. Chorismate mutase catalyzes the next step, conversion of chorismic acid to prephenic acid (prehenate), giving the basic phenyl-propanoid skeleton. Prephenic acid acts as the precursor to three aromatic amino acids: arogenic acid, tyrosine, and phenylalanine. The conversion of prehenate to arogenate is catalyzed by pyridoxal 5′-phosphate-dependent prephenate aminotransferase, and arogenate dehydratase catalyzes the subsequent step of formation of L-phenylalanine from arogenate.

12.5.1 Hydroxycinnamates

Phenylalanine is subsequently deaminated by the action of phenylalanine ammonia-lyase (PAL) producing *trans*-cinnamic acid. PAL, a tetrameric protein of 270–330 kDa, is considered as the key regulatory enzyme of the phenylpropanoid pathway.

trans-Cinnamic acid is subsequently hydroxylated by cinnamate 4-hydroxylase (C4H) to form *p*-coumaric acid. *p*-Coumaric acid further goes through a series of hydroxylation and methylation reactions to form many other hydroxycinnamates (C_6–C_3) including

caffeic acid (caffeate), ferulic acid (ferulate), 5-hydroxyferulic acid (5-hydroxyferulate), and sinapic acid (sinapate). Most often, hydroxycinnamates exist as conjugates of esters and amides. The reaction of *p*-coumaric acid to form *p*-coumaroyl-CoA is catalyzed by 4-hydroxycinnamoyl CoA ligase (4CL).

12.5.2 Hydroxybenzoates

Hydroxybenzoate (C_6-C_1), for example, gallic acid (gallate), is a common phenolic acid that is derived from hydroxycinnamates. Several pathways of biosynthesis of individual hydroxybenzoates have been proposed. Side-chain degradation of hydroxycinnamates is one of the common routes of the formation of many hydroxybenzoates. Under some conditions, gallate can also be synthesized from shikimic acid or can be a result of degradation of flavonoids. Gallate is present in some fruits as gallotannins. *p*-Coumaric acid derivatives and *p*-coumaroyl-CoA are also the precursors for lignans ($[C_6{-}C_3]2$) and lignins ($[C_6{-}C_3]n$), dimeric and polymeric phenylpropanoids, which has a significant role in the structural components of cell walls. Decarboxylation of benzoic acid and phenylpropanoid derivatives can result in the formation of simple phenols such as catechol.

12.5.3 Chlorogenic acid

The exact biosynthetic pathway of chlorogenic acid (caffeoyl quinic acid) formation in fruits is not very clear; however, biosynthesis diverges from the flavonoid biosynthetic pathway downstream of PAL, but upstream of chalcone synthase (CHS) (Fig. 12.3). Three different biosynthetic pathways of chlorogenic acid have been elucidated (Niggeweg et al., 2004). The first route diverges from *p*-coumaroyl-CoA, by formation of an ester bond with shikimic acid with the aid of the enzyme hydroxycinnamoyl transferase (HCT) to produce *p*-coumaroyl shikimic acid. This molecule is converted to caffeoyl shikimic acid by the enzyme *p*-coumarate 3′-hydrolase (C3H) (Niggeweg et al., 2004). HCT is then used again to convert caffeoyl shikimic acid to caffeoyl CoA and then to hydroxycinnamoyl CoA by quinate hydroxycinnamoyl transferase (HQT), which replaces CoA with quinic acid to produce chlorogenic acid (route 1) (Stockigt and Zenk, 1974; Niggeweg et al., 2004). Alternatively, cinnamic acid can be bound to glucose via UDP-glucose/cinnamate glucosyl transferase (UGCT) to produce cinnamoyl D-glucose. Two hydroxyl groups are then added to the cinnamoyl D-glucose by yet unknown enzyme(s) to form caffeoyl D-glucose. The glucose group is then replaced by quinic acid through the action of hydroxycinnamoyl D-glucose/quinate hydroxycinnamoyl transferase (HCGQT) to produce chlorogenic acid (Niggeweg et al., 2004) (route 2). A third theorized pathway branches off from *p*-coumaroyl-CoA, creating an ester bond to quinic acid using the enzyme HCT to produce *p*-coumaroyl quinic acid. An addition of a hydroxyl group at the carbon-3 position of *p*-coumaroyl quinic acid by C3H completes the final step for the biosynthesis of chlorogenic acid of this putative pathway (route 3).

12.5.4 Flavonoids

The step of the formation of the C_{15} aglycone skeleton (C6–C3–C6) of flavonoids is the condensation of one molecule of *p*-coumaroyl-CoA and three molecules of malonyl-CoA

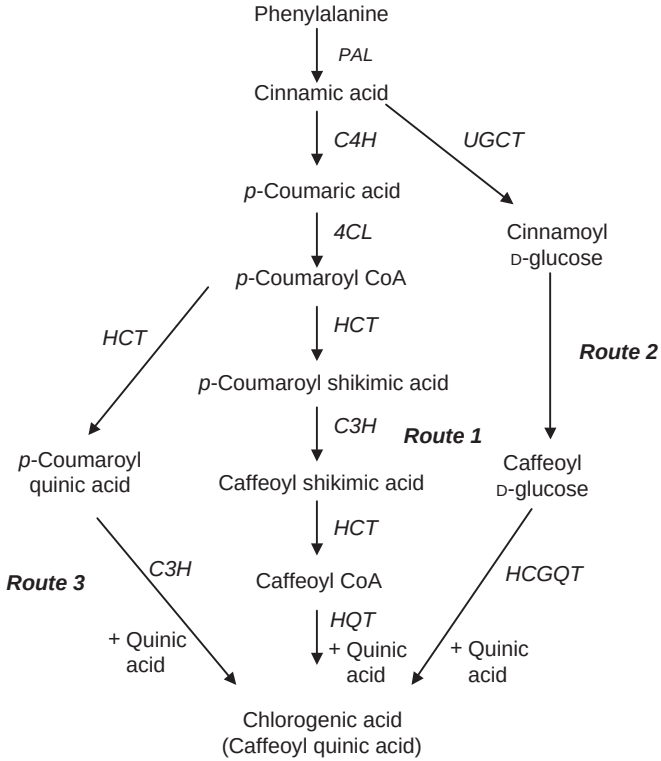


Fig. 12.3 Proposed three biosynthetic pathways for chlorogenic acid formation in plants. Enzymes as follows: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-hydroxycinnamoyl CoA ligase; HCT, hydroxycinnamoyl transferase; C3H, *p*-coumarate 3′-hydrolase; HQT, hydroxycinnamoyl CoA quinate hydroxycinnamoyl transferase; UGCT, UDP-glucose/cinnamate glucosyl transferase; HCGQT, hydroxycinnamoyl D-glucose/quinate hydroxycinnamoyl transferase. (Adapted from Niggeweg et al., 2004.)

to yield naringenin chalcone (2′,4,4′,6′-tetrahydroxychalcone) (Crozier et al., 2000) (Fig. 12.4). This rate-limiting step of the flavonoid biosynthesis is catalyzed by CHS, a dimeric protein of 78–88 kDa. Similar to the stepwise addition of acetate from malonyl-CoA to *p*-coumaroyl-CoA to form naringenin chalcone, stilbenes (C_6—C_2—C_6) such as resveratrol can be synthesized. Naringenin chalcone is isomerized to a flavanone by the enzyme chalcone isomerase (CHI). From these central intermediates, the pathway diverges into several side branches, each resulting in a different class of flavonoids: flavone, flanonol, flavanol, and anthocyanins. Once the basic C_6—C_3—C_6-carbon flavan nucleus is synthesized, numerous hydroxylation, methoxylation, and/or glycosylation reactions can occur, ultimately resulting in the synthesis of a water-soluble compound that is subsequently transported to the vacuole for deposition (Marrs et al., 1995).

Formation of flavone from flavanone by the introduction of a double bond between C-2 and C-3 is a two-step reaction catalyzed by flavone synthase (FNS) I and II. Formation of flavonols occurs from flavanone as well, through dihydroflavonol. Two enzymes involved in the two-step conversion are flavanone 3-hydroxylase (F3H) and flavonol synthase (FLS), respectively. Biosynthesis of flavan-3-ols and anthocyanidins requires the formation of an intermediate flavan-3,4-*cis*-diol (leucoanthocyanin) from dihydroflavonol catalyzed by

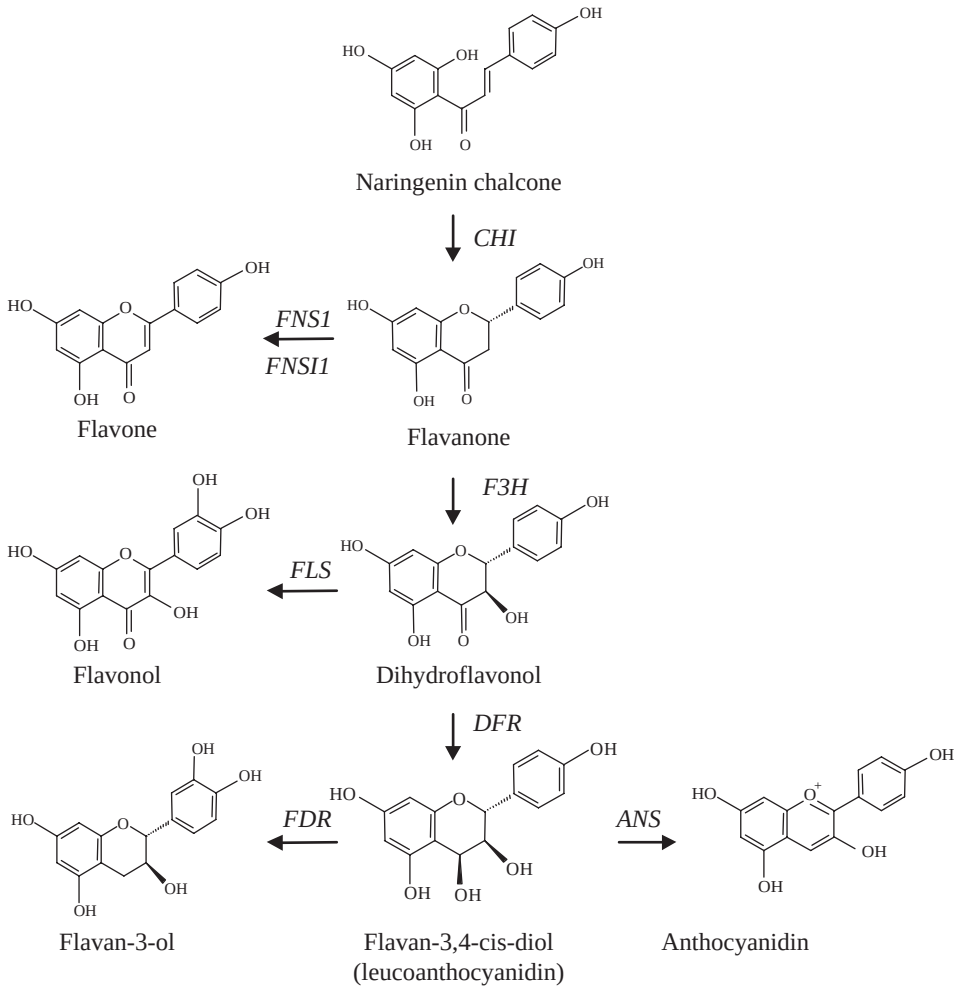
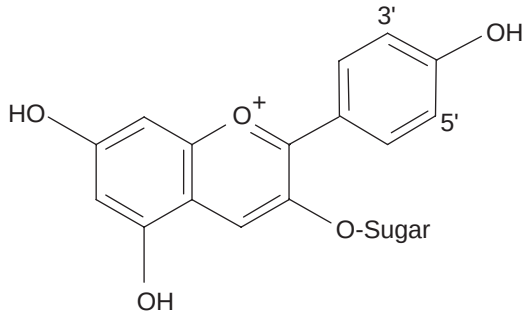


Fig. 12.4 Biosynthesis of major fruit flavonoids, flavanone, flavone, flavonol, flavan-3-ol, and anthocyanidin. Enzymes as follows: CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase; FNS, flavone synthase; FLS, flavonol synthase; ANS, anthocyanidin synthase; FDR, flavan-3,4-*cis*–diol reductase.

nicotinamide adenine dinucleotide phosphate (NADPH)-dependent dihydroflavonol 4-reductase (DFR). Anthocyanindin biosynthesis from flavan-3,4-*cis*-diol is catalyzed by anthocyanidin synthase (ANS). The key step of formation of flavan-3-ols from flavan-3,4-*cis*-diol is catalyzed by NADPH-dependent flavan-3,4-*cis*-diol reductase. Except flavan-3-ols, flavonoids usually occur in fruits as glycosides.

12.6 Regulation of flavonoid synthesis during ripening and storage

Red pigmentation in apple fruit is due to a unique anthocyanin, cyanidin-3-*O*-galactoside, which serves as an indicator of fruit maturity or ripeness for the apple producer and consumer (Fig. 12.5). Flavonoid biosynthesis, including the anthocyanins, is regulated in part by the



Anthocyanin	Substituents	Color
Pelargonidin		Orange red
Cyanidin	3′-OH	Purplish red
Delphinidin	3′-OH, 5′-OH	Bluish purple
Peonidin	3′-OCH_3	Rosy red
Petunidin	3′-OCH_3, 5′-OH	Purple
Malvidin	3′-OCH_3, 5′-OCH_3	Pink/red

Fig. 12.5 Chemical structures of selected anthocyanins of fruits. The major sugars of C-3 glycosylation include glucose, galactose, arabinose, rhamnose, and rutinose.

activity of PAL, which in turn is regulated by ethylene (Blankenship and Unrath, 1988; Gomez-Cordoves et al., 1996). Exposure of fruit to UV light in combination with low-night and high-day temperatures can also regulate anthocyanin biosynthesis (Reay, 1999). For example, it was found that both UV light and advancing maturity promoted anthocyanin biosynthesis in “Jonathan” apple (Reay and Lancaster, 2001). It has been found that the concentration of anthocyanins was higher on the blush side than on the shaded side of “Elstar” and “Jonagold” apples, and in fruit at the top and sides of the apple tree canopy (Awad and de Jager, 2000). Fine regulation of the PAL activity may be dependent on the extent of light exposure to the fruit (tree position) and climatic conditions during fruit maturation. It has also been demonstrated that UV light and low temperatures can induce the production of other flavonoids and phenolic acids (Lancaster et al., 2000). During cold storage of apple (“Granny Smith,” “Crofton,” and “Lady Williams”), flavonoid levels have been shown to remain constant (Golding et al., 2001), although some studies have reported minor fluctuations in levels. A similar study using “Jonagold” and “Elstar” cultivars found that most flavonoids and hydroxycinnamic acid derivatives were stable throughout cold or controlled atmosphere storage (Awad and de Jager, 2000). In “Delicious” and “Ralls” apple fruit, flavan-3-ols and flavonols were generally stable during storage; however, an increase in anthocyanin concentration with a concomitant decrease in simple phenols has also been reported (Ju et al., 1996). Others have reported a proportional decrease in anthocyanin concentration in storage (Lin et al., 1989). It seems that anthocyanin content remained constant during storage, while the carotenoid levels increased and chlorophyll levels decreased (Reay, 1998). It has also been suggested that factors other than the level of ethylene in the tissue contribute to the de novo biosynthesis of anthocyanins, including low temperature (Arawaka, 1991), fruit maturity (Murphey and Dilley, 1988), and storage

duration (Jiang and Joyce, 2003), all of which are important considerations in the postharvest storage of pome fruits.

12.7 Role of polyphenols in enzymatic browning during fruit processing

During postharvest storage and processing, the metabolic changes of fruit contents are mainly responsible for the changes in quality of the fruit (color, taste, texture, shelf life) or their processed products. Polyphenols and their interaction with other fruit components have a significant influence on the quality and processing of fruits (Nicoli et al., 2000). Therefore, the role of polyphenols in enzymatic browning in fruits is considered one of the most important phenomena in postharvest and fruit processing. Also, polyphenols are highly sensitive to heat, light, air, and moisture; thus, exposure to such conditions can result in significant loss of many of polyphenolic compounds, reducing the nutraceutical value of fruit products (Nicoli et al., 1999).

The exposure of the cut surfaces of certain fruits to air results in browning due to the polyphenol oxidase (PPO)-catalyzed oxidation of certain phenols to orthoquinones. The orthoquinones form dark-colored pigments or melanin due to subsequent rapid nonenzymatic polymerization (deMan, 1990). Peroxidase (POX), just as PPO may be involved in browning reactions and production of melanin compounds. The actions of PPO and POX could also result in off-flavor generation in horticultural products. Both phenomena are of vital importance to the manufacturer as they impair not only the sensory properties and marketability of a product, but also lower the nutritional value of fruit. The activity of PPO and POX can significantly reduce the quality of the fruits after harvesting. Postharvest browning losses in litchi resulted from the oxidation of phenolics by PPO and POX enzymes (Zhang and Quantick, 1997). The presence of active PPO in plant tissues can also cause significant loss of anthocyanins and result in the production of quinones and loss of color, flavor, and nutritive value (WescheiEbeling and Montgomery, 1990; Kader et al., 1998).

PPO belongs to the group of enzymes called oxidoreductases. PPO is present in almost all the plants, but the enzyme is most abundant or active in fruits such as apples, peaches, banana, and avocados. The distribution of PPO in the different parts of fruits may be considerably different, and the ratio of particle-bound and soluble enzymes varies with maturity. In fruits such as pears and apples, PPO was found to be distributed in almost all of the fruit parts (Vamos-Vigyazo, 1981). A wide range of PPO is characterized from plants with characteristic substrate (phenolic compounds) specificity, for example, tyrosinase, cresolase, phenolase, catechol oxidase or *o*-diphenol oxidase, laccase, or *p*-dipenol oxidase. (Sapers, 1993; Martinez and Whitaker, 1995). PPO catalyzes two basic reactions: hydroxylation at the *o*-position adjacent to an existing hydroxyl group of the phenolic substrate (monophenol oxidase activity) and oxidation of diphenol to *o*-benzoquinones (diphenol oxidase activity) (Fig. 12.6). Both reactions utilize molecular oxygen as a cosubstrate. The protein copper–oxygen complex is formed by combining one molecule of oxygen with the protein to which two adjacent cuprous atoms are attached.

All PPOs possess catecholase activity, that is, they can convert *o*-dihydroxyphenols to *o*-benzoquinones, but not all PPOs can oxidize monophenols. However, much attention is paid to the diphenol oxidases due to their high catalytic rate and their role in the production of the brown pigment, melanin. Most importantly, once *o*-quinones are formed, they can undergo

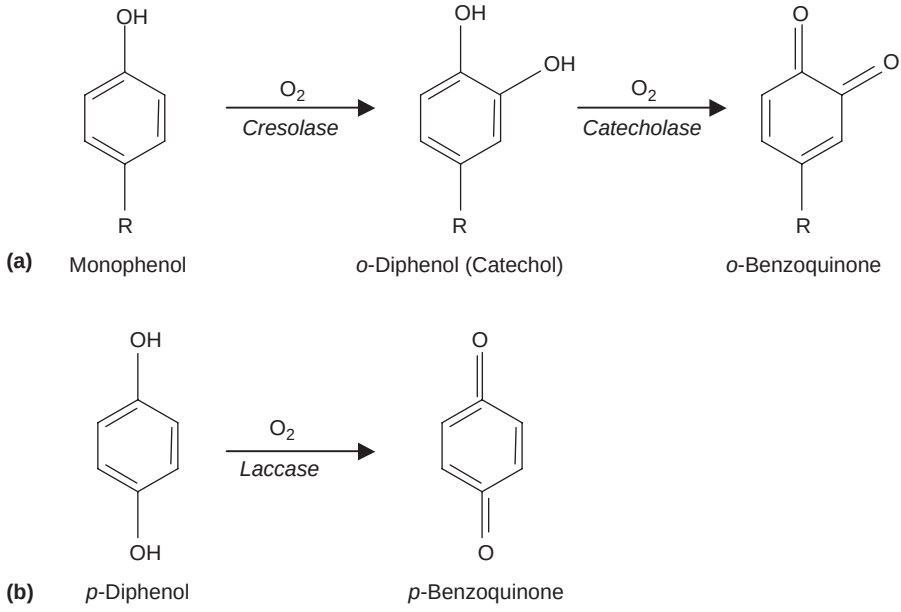


Fig. 12.6 Polyphenol oxidation reactions catalyzed by polyphenol oxidase (PPO): (a) monophenol oxidation pathway catalyzed by cresolase and *o*-diphenol oxidase (catecholase); and (b) *p*-diphenol oxidation catalyzed by *p*-diphenol oxidase (laccase). (Adapted from Marshall et al., 2000.)

secondary reactions (nonenzymatic reactions) to form higher-molecular-weight polymers; to form macromolecular complexes with amino acids or proteins; and to oxidize compounds of lower oxidation–reduction potentials (Vamos-Vigyazo, 1981). The third types of reactions are considered to be most destructive as quinones can be reduced back to dihydroxyphenols. Hence, these continue to provide fresh substrate for PPO until it gets inactivated by reaction products or the compounds of lower oxidation–reduction potentials such as ascorbic acid that gets depleted (Pifferi and Cultrera, 1974). This is one of the mechanisms responsible for the inhibitory action of ascorbic acid in enzymatic browning (Baruah and Swain, 1953; deMan, 1990; Ozdemir, 1997). Laccase is also a type of PPO, which has the unique ability of oxidizing *p*-diphenols, which is not shown by *o*-diphenol oxidases such as catechol oxidase; however, laccase does not act on monophenols. It is less frequently encountered in fruits and vegetables except some peach cultivars (Harel et al., 1970), mushrooms, and tomatoes.

POX, just as PPO, belong to the same group of enzymes, oxidoreductases (Vamos-Vigyazo, 1981). POX is widely distributed in nature and catalyzes the decomposition of hydrogen peroxide (H_2O_2) in the presence of a hydrogen donor. A great variety of compounds may act as hydrogen donors, including phenols (*p*-cresol, guaiacol, and resorcinol), aromatic amines (aniline and benzidine), reduced nicotinamide-adenine dinucleotide, and reduced nicotinamide-adenine dinucleotide phosphate (Vamos-Vigyazo, 1981). It has been observed that the wounding of fruits results in an increase of POX activity besides PPO activity (Cantos et al., 2002). The generation of H_2O_2 by POX during the oxidation of phenolics catalyzed by PPO also suggests the role of POX in enzymatic browning processes (Subramanian et al., 1999). However, the content of H_2O_2 is very less in the plant tissue, but

these enzymes along with PPO, catalase, and lipoxygenase may result in quality losses by inducing changes in the flavor, color, texture, and nutrient value of horticultural commodities. The reactions catalyzed by POX are of four types: peroxidative, oxidative, catalytic, and hydroxylation.

The overall equation can be given as follows:

$$ROOH + AH_2 \rightarrow 2H_2O + ROH + A$$

where R = H^+, CH_3, or C_2H_5; AH_2 = hydrogen donor in the reduced form; and A = hydrogen donor in the oxidized form.

The oxidative reaction of POX may take place in the absence of H_2O_2, but for this, it requires O_2 and cofactors—Mn^{2+} and a phenol (mostly 2,4-dichlorophenol) (Kay et al., 1967). The catalytic decomposition of H_2O_2 occurs in the absence of a hydrogen donor as per the following equation:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

The rate of this reaction is negligible as compared to the rates of the peroxidative and the oxidative reactions (Vamos-Vigyazo, 1981). The hydroxylation reaction produces *o*-dihydroxy phenols from monophenols and O_2.But the reaction requires a hydrogen donor, for example, dihydroxyfumaric acid, which provides free radicals necessary for the enzyme action.

12.7.1 Factors important for enzymatic browning

The most important factors that determine the rate and intensity of enzymatic browning are the activity of enzyme, concentration of specific polyphenols present in the tissue, oxygen availability, the pH, and the temperature (Martinez and Whitaker, 1995). Browning reactions are also dependent on the mechanical integrity of cell membranes (Dornenburg and Knorr, 1997). In addition to this, the subsequent nonenzymatic browning also influences the PPO activity. The optimum pH and temperature for PPO activity varies with the source of the enzyme and the substrate (Vamos-Vigyazo, 1981). Thermotolerance of the PPO depends on the substrate specificity, pH, and also the source of the enzyme. Short exposures of the tissues to a temperature range of 70–90°C are sufficient for partial or complete destruction of PPO activity. Enzymatic browning in fruits, for example, apples, can be controlled by blanching, a pretreatment, which results in inactivation or destruction of PPO. In a recent study, it has been found that low-temperature long-time (LTLT) treatment at 75°C for 5 min and high-temperature short-time (HTST) treatment at 90°C for 10 s is sufficient to control the enzymatic browning in apple (Rupasinghe et al., unpublished). However, the thermal treatment may result in a loss of phenolic compounds, vitamins, and other water-soluble nutrients and also affect the product quality such as flavor, color, taste, and texture (Biekman et al., 1996). To prevent these changes, nonthermal methods such as application of chemical inhibitors have been devised for inactivation of PPO (Sapers et al., 1990; Sisler and Serek, 1997; Son et al., 2001; Rupasinghe et al., 2005). The enzyme activity gets inhibited by the presence of acids, halides, phenolic acids, sulfites, chelating agents, and reducing agents such as ascorbic acid, quinine couplers such as cysteine, and various substrate-binding

compounds. The role of metal ions influencing the enzymatic browning has also been investigated by several researchers. According to Aydemir (2004), Cu^{2+} and Fe^{3+} ions at 1 mM caused the activation of PPO, but at 10 mM concentration, both Cu^{2+} and Fe^{3+} ions acted as poor inhibitors of PPO. Whereas Colak et al. (2007) and Kolcuoglu et al. (2007) have recently found that Cu^{2+} at 1 mM concentration was sufficient to inhibit PPO activity. As well, the reported literature on the effects of different metal ions on the PPO activity is varying. Interestingly, a strong correlation ($r^2 = 0.92$) between copper content of fruit and PPO activity has been reported (Joshi et al., 2007). However, further investigations need to be carried out to understand the role and concentration-dependent effect of metal elements on PPO activity and enzymatic browning.

The pH optimum of POX varies with the enzyme source, the isoenzyme composition, and the hydrogen donor substrate. In fruits it generally ranges from pH 4.0 to 6.5 (Vamos-Vigyazo, 1981). The behavior of POX during different heating and cooling treatments is most widely investigated due to the existence of different fractions of its heat resistance, and part of its activity is restored during shorter or longer storage at room temperatures following the thermal treatment.

12.7.2 Polyphenols and substrate specificity for PPO and POX

The substrate specificity of PPO varies in accordance with the source of the enzyme. The extent to which naturally occurring phenolic substrates contribute to enzymatic browning of individual fruits depends on the localization and concentration of phenolics as well as on the color intensity of the macromolecular pigments obtained from the different quinones (Vamos-Vigyazo, 1981). For example, total phenolic content among apple cultivars is highly variable (Lee et al., 2003; Lata et al., 2005; Scalzo et al., 2005), which is the cause of the differences in the browning intensity among cultivars (Russell et al., 2002). A wide range of phenolic compounds is oxidized by PPO, and hence there is a high potential for browning besides 3,4-dihydroxyphenylalanine (DOPA) and tyrosine (Baruah and Swain, 1953; Sapers, 1993). The phenolic substrates of PPO in different fruits are given in Table 12.3.

Among phenolic compounds, catechin and chlorogenic acids are the substrates with a greater affinity for PPO enzyme activity (Janovitz-Klapp et al., 1990; Oszmianski and Lee, 1990), whereas the flavonols appear to be less suitable as PPO substrates (Baruah and Swain, 1953). However, in the presence of transfer substances such as chlorogenic acid and catechin, flavonols glycosides are oxidized at measurable rate, probably through the formation of dimers as a first step (Vamos-Vigyazo, 1981). However, in certain fruits and vegetables, the main substrates of PPO are not catechin and chlorogenic acid (Marshall et al., 2000). The principal phenolic substrate in banana, for example, was identified as dopamine (3,4-dihydroxy phenylethylamine), while that in dates is 3-*O*-caffeoylshikimic acid (dactylifric acid). The catechins oxidized more rapidly as compared to others; however, the higher concentration of chlorogenic acid in apples is considered to play a decisive role in acting as PPO substrate. Based on the degree of browning of 11 apple cultivars subjected to bruising, Amiot et al. (1992) found that chlorogenic acid and catechins are the most degraded phenolics as a result of enzymatic browning. However, in a recent study, it was observed that "Eden™," an apple cultivar that contains reasonable amount of chlorogenic acid but very low level of catechin and epicatechin, did not brown as compared to "Empire"

Table 12.3 Different phenolic substrates of PPO in fruits

Fruit	Polyphenolic substrates for PPO
Apple	Chlorogenic acid, catechol, catechin, caffeic acid, 3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxy benzoic acid, *p*-cresol, 4-methyl catechol, leucocyanidin, *p*-coumaric acid, flavonol glycosides
Apricot	Isochlorogenic acid, caffeic acid, 4-methyl catechol, chlorogenic acid, catechin, epicatechin, pyrogallol, catechol, flavonols, *p*-coumaric acid derivatives
Avocado	4-Methyl catechol, dopamine, pyrogallol, catechol, chlorogenic acid, caffeic acid, DOPA
Banana	3,4-Dihydroxyphenylethylamine (dopamine), leucodelphinidin, leucocyanidin
Grape	Catechin, chlorogenic acid, catechol, caffeic acid, DOPA, tannins, flavonols, protocatechuic acid, resorcinol, hydroquinone, phenol
Mango	Dopamine-HCl, 4-methyl catechol, caffeic acid, catechol, catechin, chlorogenic acid, tyrosine, DOPA, *p*-cresol
Peach	Chlorogenic acid, pyrogallol, 4-methyl catechol, catechol, caffeic acid, gallic acid, catechin, dopamine
Pear	Chlorogenic acid, catechol, catechin, caffeic acid, DOPA, 3,4-dihydroxy benzoic acid, *p*-cresol
Plum	Chlorogenic acid, catechin, caffeic acid, catechol, DOPA

Adapted from Marshall et al. (2000).

and "Cortland." This shows that catechin and epicatechin might play more decisive role than chlorogenic acid in causing enzymatic browning (Joshi et al., 2007). The site of substitution of mono- and dihydroxy phenols is also an important factor from the aspect of the action of PPO. Monophenols are hydroxylated only if they have a parasubstituted CH_2 group, and *p*-substituted 3,4-dihydroxy phenols are oxidized at higher rates than 2,3-dihydroxy phenols (Baruah and Swain, 1953).

On the other hand, POX is highly specific to the peroxide substrate, and its main substrate is H_2O_2. POX has low specificity for hydrogen donor substrates. POXs are able to oxidize hydroxycinnamic derivatives and flavans. They can also oxidize flavonoids, which are not PPO substrates but are found degraded in brown fruits due to progressing of coupled oxidation reactions.

In addition to serving as PPO substrates, some phenolic compounds can also act as inhibitors of PPO. For example, certain flavonoids, cinnamic acid derivatives, and coumarins are shown to inhibit PPO activity. Quercetin is not a preferred substrate for PPO, but act as a competitive inhibitor of PPO (Xie et al., 2003). Another flavonol, kaemferol, has also been found to inhibit PPO, presumably through a mechanism of chelating copper in the enzyme (Kubo and Kinst-Hori, 1999). Natural and synthetic naphthoquinones were found to inhibit POX activity. Some of them showed a stimulating effect in a certain concentration range (Vamos-Vigyazo, 1981).

12.7.3 Enzymatic browning of minimally processed fruits

Enzymatic browning has direct influence on the color, flavor, and sensory attributes of fresh as well as processed fruit products. Minimally processed fruits are also called as fresh-cut, lightly processed, partially processed, or ready-to-eat products, designed to keep the produce fresh without losing the convenience and nutritional quality, and also to have an increased shelf life (Lattanzio, 2003). In general, the processing steps of these products include

washing, trimming or slicing, shredding, and packaging. Fresh-cut fruit may undergo surface browning, tissue softening, loss of flavor, and other deterioration reactions. Surface discoloration is considered to be the most important quality defect and the factor mostly limiting shelf life of fresh-cut fruits (Lule and Xia, 2005; Toivonen, 2006). During peeling and cutting, fruit cells are ruptured and thus, PPO located in the cytosol comes in contact with phenolic substrates that are stored in the vacuole. Furthermore, secondary reactions results in the formation of complexes between quinones and proteins causing changes in physical, chemical, and nutritional characteristics, and reducing the shelf life of the fresh-cut fruit products (Lindley, 1998). Also, these reactions in the advanced phases produce strongly oxidized phenolic compounds with a subsequent loss of antioxidant capacity of these compounds (Nicoli et al., 2000). In addition, PAL, a key enzyme in the phenolic synthesis of phenylpropanoid biosynthesis, can be stimulated (Lattanzio, 2003) after wounding or injury. As mentioned earlier, cutting or bruising of minimally processed fruits also increases the activity of POX.

Control of enzymatic browning can be achieved through the use of physical (reduction of temperature and oxygen, and use of modified atmosphere packaging or edible coatings) and chemical methods (treatment with compounds that inhibit PPO, remove its substrates (oxygen and phenolics) or, function as preferred substrates). There has been an extensive research on investigating ways to prevent or minimize enzymatic browning, especially since the banning of the use of sulfites to prevent the browning of fresh-cut fruits and vegetables. Commercial antibrowning agents such as FreshXtend^{TM} (FreshXtend Technologies Corp., Vancouver, British Columbia, Canada) and NatureSeal^{TM} (Mantrose-Haeuser Co. Inc., CT) are available in the market, but their high cost is a limitation for their application in many value-added fruit-processing operations. In addition to search for low cost and environmentally friendly antibrowning agents, the development of cultivars with a low potential for enzymatic browning can significantly contribute toward the prevention of browning in minimally processed fruits.

12.7.4 Enzymatic browning of fruit juices

Juice and beverage products represent a significant portion of the processed fruit industry. However, the enzymatic browning caused by fruit pressing and juice extraction is the most critical step that imparts unfavorable quality to most fruit juices (Macheix et al., 1991). Processing steps such as cutting, crushing, and pressing enhance PPO activity (Lozano et al., 1994). Therefore, it is recommended that juice extraction should be done as quickly as possible and passed through filtration process to the pasteurization step, which can inactivate PPO enzyme. Also, crushed fruit can be passed through larger bore, tubular heat exchanger (50–60°C) to minimize enzymatic browning. The application of high-intensity pulsed electric fields (PEF) treatments has been also investigated by some researchers. The PEF treatment depleted PPO and POX activities of grape juice although it was observed that grape POX was less sensible than PPO to PEF technology (Marselles-Fontanet and Martin-Belloso, 2007).

In certain cases, PPO is considered beneficial for oxidation of phenolics as it may help reduce the haze problem in juices. Oxidation of phenolic compounds such as procyanidins can generate highly reactive intermediates that can complex irreversibly with each other and with proteins to form insoluble complexes, which are not dissolved on warming

(Lea and Timberlake, 1978; Spanos and Wrolstad, 1992). Therefore, oxidation of juice, which contains high levels of procyanidins, tends to promote formation of haze. An early oxidation of juice in the presence of pulp may prevent the formation of haze in the finished juice products (Pilnik and deVos, 1970; Spanos and Wrolstad, 1992). In this case, oxidation of procyanidins leads to the tanning of the pomace and significant removal of these constituents. However, excessive pulp oxidation can reduce the color and flavor of apple juice (Lea and Timberlake, 1978). In the case of fermented pear juice and cider, the condensed and polymerized oxidation products of phenolics result in sediments that can be removed by filtration to reduce the astringency of the beverages (Vamos-Vigyazo, 1981).

12.7.5 Control of enzymatic browning by regulation of phenylalanine ammonia-lyase activity

Regulation of phenylalanine ammonia-lyase (PAL), the key regulatory enzyme of shikimic acid pathway that catalyzes the conversion of phenylalanine to *trans*-cinnamic acid, plays a pivotal role in phenolic synthesis. The correlation between increases in the corresponding PAL gene/protein expression/activity and increases in phenolic compounds in response to different stimuli has been well established (Hahlbrock and Scheel, 1989). Control of PAL activity, and thereby the biosynthesis of phenolic compounds at the site of injury to the fruit, is also important in controlling enzymatic browning caused by postharvest and processing steps (Martinez and Whitaker, 1995). Activity of PAL is stimulated under conditions such as wounding, light, low temperature, and pathogens, thereby, the PAL activity regulates the phenyl propanoid pathway. There are some other enzymes that may also participate in increasing the phenolic production during cold storage such as C4H, CQT, and 4CL. Hence, the increased content of phenolics provides an opportunity for PPO resulting in more browning. It has also been reported in some fruits that the increase in PAL activity takes place during cold storage, but PPO activity remains the same. This suggests that the accumulated phenolics might undergo nonenzymatic reactions in the presence of metal ions such as iron stored as ferritin (Lattanzio, 2003).

12.8 Other biochemical regulation of phenyl propanoid pathway

It has been observed that differential subcellular distributions of cinnamic acid arising from the activities of differentially localized PAL isoforms could partition phenylpropanoid biosynthesis into different branch pathways including those that bypass the C4H reaction (e.g., 2-hydroxylation of cinnamic acid) (Achnine et al., 2004). Interestingly, this work suggests that changes in the subcellular location of enzyme isoforms might provide an extra and unsuspected level of metabolic regulation. More than 16 cytochrome P450 monooxygenases have been recently shown to be involved in all these branch pathways. Downregulation of C4H in transgenic plants (Reddy et al., 2005) and C4H mutation (Ruegger and Chapple, 2001) resulted in reduction in chlorogenic acid, flavonoid, and lignin biosynthesis, which proves that C4H constitutes a rate-limiting step for channeling carbon flux into the phenyl propanoid pathway (Anterola et al., 2002). Recently, Miziak et al. (2007) found that 2-amino-4-bromoindane-2-phosphoric acid acts as a potent inhibitor of PAL activity in vitro and of anthocyanin biosynthesis in vivo. Cinnamaldehyde was also shown to inhibit PAL

(Fujita et al., 2006), and interestingly this compound suppresses the browning of cut lettuce when they are immersed in a solution of the inhibitor. Other chemical compounds such as 1-methylcyclopropene (1-MCP) dramatically inhibits ripening of apple fruit (Rupasinghe et al., 2000). 1-MCP interacts with ethylene receptors and thereby prevents ethylene-dependent responses (Rupasinghe et al., 2000). Action of 1-MCP results in decreasing the PAL and PPO activity, and also lowering the phenolic content. Its application also prevents or delays the softening, the effects of treatment often closely associated with ethylene production (Rupasinghe et al., 2000). In apple, banana, melon, and pear fruit, inhibition of ethylene production by 1-MCP was accompanied by lower expression of these genes (Defilippi et al., 2005). Hexanal can also act as possible inhibitor of PAL (Lanciotti et al., 2004).

12.9 Selection for apple genotypes with low postcut enzymatic browning

New genotypes with antibrowning properties have a growing demand for value-added product development for niche markets while minimizing the processing cost by eliminating the use of antibrowning chemical treatments. Several research programs are presently attempting to develop fruits exhibiting low enzymatic browning (Martinez and Whitaker, 1995; Marshall et al., 2000). Conventional breeding and genetic engineering methods, such as antisense RNA and gene silencing techniques, have been investigated as means to develop new genotypes with antibrowning properties (Martinez and Whitaker, 1995). One possible approach to reduce the PPO activity and resultant enzymatic browning reactions is to characterize and inactivate the genes, which code for PPO enzyme. Inactivation can be accomplished by generating antisense RNAs specific for PPO. Expression of PPO mRNA might be controlled in this way, a reduction in browning would be accomplished by reducing the amount of protein formed (Martinez and Whitaker, 1995). Genetically modified versions of "Golden Delicious" and "Granny Smith" apple fruit have been produced in which 95% of PPO was silenced by the antisense insertion of polyphenol oxidase genes derived from apple (Okanagan Biotechnology Inc., 2005). Eden, "SuperMac," and SJCA16R5A15 are some of the recently developed apple genotypes using conventional breeding and selection by Agriculture and Agri-Food Canada—Horticultural Research and Development Center (AAFC-HRDC), Quebec, Canada. Two hours after slicing and storage at ambient temperature, the whiteness index (WI) values were significantly higher for Eden (Fig. 12.7) as compared to commercial apple genotypes tested (Joshi et al., 2007). Similar results were observed for Eden after vacuum dehydration (50°C for 24 h), while the WI of "Cortland" and SJCA16R5A15 was greater than "Empire" and "SuperMac." These results suggested that Eden offers a potential nonbrowning or minimal-browning characteristic, which may make it favorable for use in processing apples for either fresh-cut or dried chips. Similar results were obtained when a range of apple cultivars including "Gala," "Galarina," "Spartan" and "Cortland," and Eden were cut and kept for 24 h at 20°C (Khanizadeh et al., 2006). However, the major concern in selecting these cultivars is that these are low in polyphenolics and hence reduce the nutritional quality and antioxidant capacity of selected cultivars. For instance, Eden showed the least total antioxidant capacity as estimated by ferric-reducing ability of plasma and oxygen radical absorbance capacity assays by comparison to other cultivars.

Fig. 12.7 Eden^{TM}, a new apple cultivar with low postcut enzymatic browning (left), is compared with MacSpur (right). The flesh of Eden contains very low amount of catechin and epicatechin, polyphenolic substrates of polyphenol oxidase (PPO) (Joshi et al., 2007). (The photograph provided by Dr S. Khanizadeh, AAFC, Canada.)

12.10 Concluding remarks

Understanding of the phenolic profiles and biosynthesis of phenolics in fruits as well as their role in postharvest biology and food processing is very important for developing high-quality fruit products. For example, understanding how polyphenolics are involved in the mechanism of enzymatic browning will help in developing efficient protocols for the control of enzymatic browning as well as to establish new approaches for development of antibrowning genotypes. Beyond the classical role of polyphenols in determining physicochemical quality of fruit and fruit products, their importance as nonnutrient health-promoting constituents as well as nontraditional natural products in various industrial applications has become one of the major focus of polyphenolic research in fruits.

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Chapter 13

Isoprenoid Biosynthesis in Fruits and Vegetables

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13.1 Isoprenoids in plants

The isoprenoid pathway provides the largest class of secondary metabolic products (over 25,000) found in higher plants, and include fragrance components (monoterpenes such as geraniol, nerol, and citronellol), sterols (sitosterol, stigmasterol, etc.), carotenoids (lycopene, xanthophylls), tocopherols, hormones (gibberellins, abscisic acid, brassinosteroids, certain cytokinins), phytyl side chain of chlorophyll, phylloquinone, plastoquinone, ubiquinone, and phytoalexins (Bach, 1995; Chappell, 1995; McGarvey and Croteau, 1995; Eisenreich et al., 2001). Isoprenoids are composed of C_5 isoprene units and are classified as hemi-(C_5), mono-(C_{10}), and sesquiterpenes (C_{15}) as well as di-(C_{20}), tri-(C_{30}), and tetraterpenes (C_{40}), and also polyterpenes. Higher polymers are encountered in materials such as rubber.

Recently, various isoprenoids have attracted commercial interests as nutraceuticals or biologically active agents with potential health benefits. For example, carotenoids form one of the most important classes of plant pigments and play a crucial role in defining the quality parameters of fruits and vegetables. In general, carotenoids such as lycopene and lutein are profound antioxidants and potential anticancer agents, and β-carotene possesses provitamin A activity. A diterpene, paclitaxel (Taxol), has been established as a major cytostatic agent (Eisenreich et al., 2001) and is used for the treatment of certain cancers. Triterpene saponins present in legumes have several biological functions including hypocholesterolemic, immunostimulatory, antioxidant, antidiabetic, and antitumor properties. Plant sterols such as sitosterol, stigmasterol, and campesterol have a similar structure to cholesterol, and have the capacity to lower plasma cholesterol and LDL cholesterol levels (Piironen et al., 2000).

13.2 Carotenoid accumulation in fruits

The development of red pigmentation is one of the most recognizable features of ripening in most tomato fruits. The major carotenoids that accumulate in ripe red tomato fruits are lycopene ($\sim$90%), β-carotene (5–10%), and lutein (1–5%), with trace amounts ($<$1%) of other carotenoids (Ronen et al., 1999). Lycopene and β-carotene are the main pigments responsible for the characteristic color of ripe fruits, conferring deep red and orange colors, respectively. These carotenoids largely influence the quality perception of fresh tomatoes

and their processed products because consumers prefer tomatoes and products with intense red color. As well, the carotenoids are nutraceuticals because they play a beneficial role in regulating health. Inverse associations have been observed between blood lycopene levels and the risk of cancer development at several sites in the human body (Giovannucci, 1999). The most beneficial effects have been observed in the reduction of the incidence of prostate, lung, and stomach cancers, with slightly lower benefits at other sites that include pancreas, colon, rectum esophagus, oral cavity, and cervix (Giovannucci, 1999). β-Carotene (provitamin A) is a vitamin A precursor, and considerable efforts have been made to increase dietary levels of β-carotene by engineering the genes for the carotenoid biosynthetic pathway enzymes into rice endosperm (Ye et al., 2000). Vitamin A deficiency causes xerophthalmia and blindness (Mayne, 1996) and has led to over a million deaths annually among children 1–4 years of age (Humphrey et al., 1992). Finally, levels of dietary lutein (Seddon et al., 1994; Curran-Celentano et al., 2001) and serum lutein (EDCCSG, 1992, 1993) are related to a reduced risk of age-related macular degeneration. For these reasons, there is a tremendous interest among the industry and grower sectors to obtain tomatoes with the highest carotenoid levels possible.

13.3 Carotenoid biosynthesis

Carotenoids are biosynthesized by the chloroplast-localized isoprenoid pathway. The accumulation of lycopene (C_{40}) during fruit ripening results from increased lycopene synthesis as well as decreased conversion of lycopene to more complex carotenoids—with the notable exception being the formation of small amounts of β-carotene at the expense of lycopene (Ronen et al., 2000). At least two enzymes are known to regulate carotenoid synthesis: 1-deoxy-D-xylulose-5-phosphate synthase (DXS) and phytoene synthase (PSY, which is coded by *PSY1* in ripe fruit). DXS appears to be the paramount of several enzymes involved in the synthesis of isopentenyl pyrophosphate, which is the key 5-carbon unit of the isoprenoid pathway (Fig. 13.1). Within this pathway, PSY forms phytoene (C_{40}), which is an immediate precursor to lycopene (Fig. 13.2). Increased expression of *DXS* and *PSY1* genes during ripening correlates with the pattern of lycopene accumulation, thus demonstrating transcriptional regulation of the pathway during ripening (Lois et al., 2000). As expected, PSY activity also correlates with these patterns of mRNA transcription and lycopene levels (Fraser et al., 1994). The accumulation of lycopene in tomato fruit has been found to be regulated by light, and this is mediated by phytochromes (Alba et al., 2000a). Phytochromes are light receptors involved in responses regulated by red light (R) and far-red light (FR) resulting in alteration of growth and development. In particular, these modified proteins change conformations depending on whether they have received R or FR; however, it is R that activates the protein within the cell to cause physiological responses (Quail, 2002). Most phytochrome responses occur at low-photon fluence rates (1–1,000 μmol/m^2) once a given number of photons have been intercepted, irrespective of the duration of the light exposure (Neff et al., 2000). As well, they are commonly characterized by R/FR reversibility.

13.4 Composition of tomato pigments

The ripening of tomato fruit is characterized by a large shift in pigment composition. Pigmentation of immature and mature green fruit is similar to that of other photosynthetic

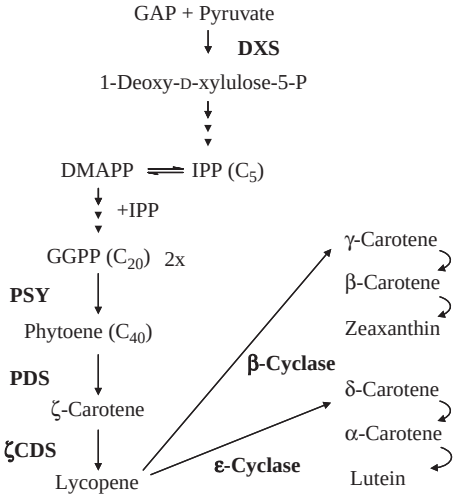

Fig. 13.1 Plastid-localized DXP/isoprenoid pathway in plants. Some key enzymes (bold face) and intermediates involved in isoprenoid biosynthesis are abbreviated: β-cyclase, ε-cyclase; ζCDS, ζ-carotene desaturase; DMAPP, dimethylallyl pyrophosphate; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; GA-3P, glyceraldehyde-3-phosphate; GGPP, geranylgeranyl pyrophosphate; IPP, isopentenyl pyrophosphate; PDS, phytoene desaturase; PSY, phytoene synthase.

tissue (i.e., the leaves): chlorophyll accounts for the green color that masks the effects of most other chromophores. The major role of the carotenoids in these tissues is to act as light receptors and protectors of the photosynthetic apparatus.

The major carotenoids that accumulate in ripe tomato fruits are lycopene ($\sim$90%), β-carotene (5–10%), and lutein (1–5%), with trace amounts ($<$1%) of other carotenoids (Ronen et al., 1999). Changes from immature green to firm red tomato fruit (4 days post-breaker, d.p.b.) are characterized by a loss of chlorophyll and a 6.7-fold increase in total carotenoids (Fraser et al., 1994). This is attributed to a 184-fold increase in lycopene and a 7.7-fold increase in β-carotene. The trend continues, and overripe fruits (14 d.p.b) show 12-fold and 282-fold increases in total carotenoids and lycopene, respectively. These changes in the carotenoid profile result from the net upregulation of *lycopene* biosynthetic genes and downregulation of the *cyclase* genes that convert lycopene to other carotenoids.

13.5 Carotenoid biosynthesis

As previously mentioned, the ripening phase of tomato fruit is marked by the loss of chlorophyll and the biosynthesis and accumulation of carotenoids, particularly lycopene (C_{40}). As these changes occur within the plastid organelles, the green chloroplasts become red chromoplasts. All plastid carotenoids are formed via the plastid-located isoprenoid pathway (Lichtenthaler, 1999) (Figs 13.1 and 13.2). The basic C_5 isoprenoid units are derived from common C_3 intermediates via another plastid-localized pathway: the 1-deoxy-D-xylulose-5-phosphate (DXP) pathway (Rohmer, 1999). This pathway is distinguished from the cytosolic pathway that generates C_5 isoprenoid units which is regulated by 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) (Bach et al., 1999; Newman and Chappell, 1999). In plants, compartmentalization of the chloroplasts prevents mixing

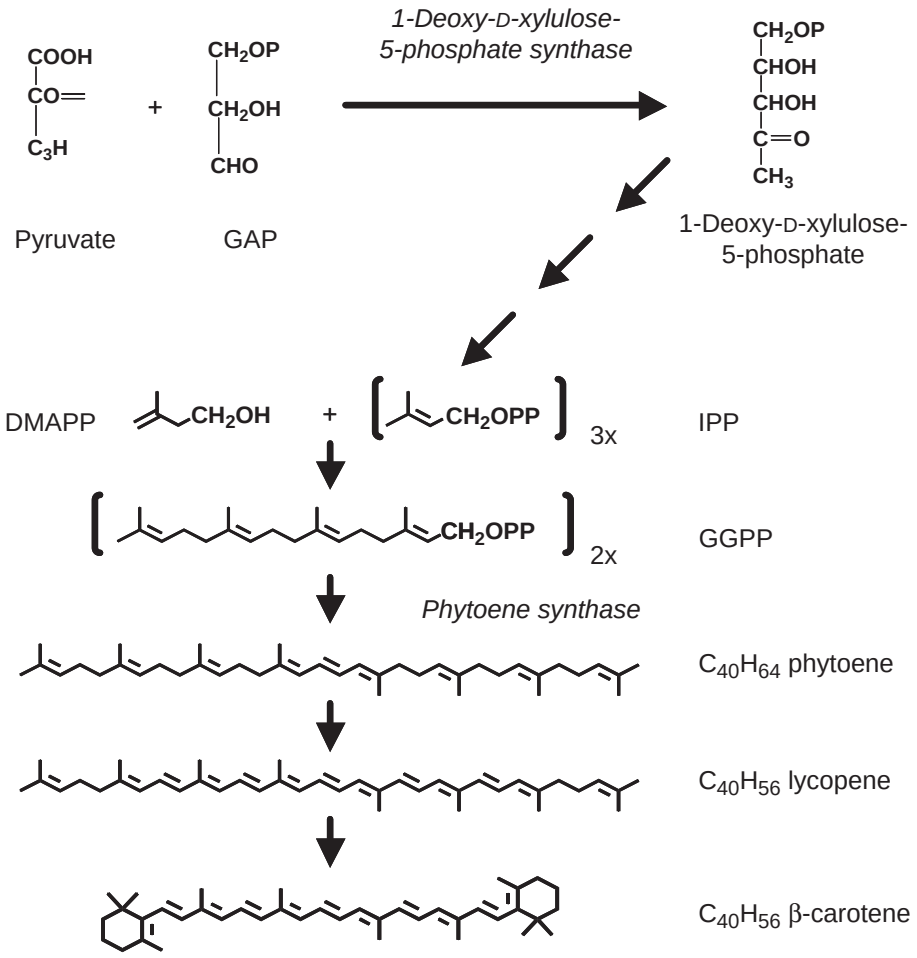

Fig. 13.2 Structure of major plastid-localized DXP/isoprenoid intermediates. Some key enzymes (bold face) and intermediates involved in isoprenoid biosynthesis are abbreviated: DMAPP, dimethylallyl pyrophosphate; GAP, glyceraldehyde-3-phosphate; IPP, isopentenyl pyrophosphate.

of plastid- and cytosolic-derived isoprenoid intermediates (Lichtenthaler, 1999; Rohmer, 1999; Eisenreich et al., 2001). This is an important point because it means that the entire carotenoid biosynthetic process—from simple C_3 molecules to complete C_{40} structures—takes place within the plastids and is regulated by a set of plastid-localized enzymes (Fig. 13.3).

DXP is derived from glyceraldehyde-3-phosphate (GAP) and pyruvate through a reaction catalyzed by the enzyme DXP synthase (DXS). The reaction involves the condensation of hydroxyethyl thiamine (derived by the decarboxylation of pyruvate) with the aldehyde group of GAP to form DXP (Lange et al., 1998; Rohmer, 1999). Through several subsequent enzymatic steps, DXP is converted to isopentenyl pyrophosphate (IPP) and its isomer, dimethylallyl pyrophosphate (DMAPP), both C_5 compounds serve as the basic building blocks of the isoprenoid pathway (Charon et al., 2000; Rodríguez-Concepción et al., 2000). Geranylgeranyl pyrophosphate (GGPP; C_{20}) is formed by GGPP synthase via the

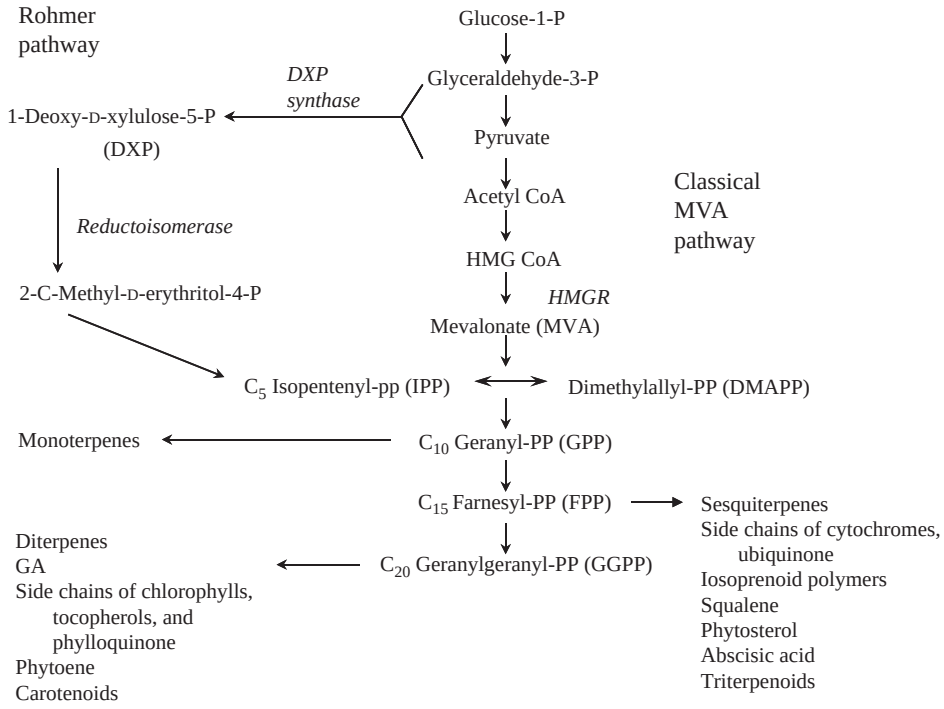

Fig. 13.3 Interrelationship between the mevalonate pathway and the DXP pathway.

sequential and linear addition of three molecules of IPP to one molecule of DMAPP. In the next step, phytoene synthase (PSY) catalyzes the first committed step of carotenoid biosynthesis: the condensation of two molecules of GGPP to form phytoene (C_{40}) (Cunningham and Gantt, 1998). Phytoene is converted to lycopene via four desaturation reactions. The first two are catalyzed by phytoene desaturase (PDS) and the second two are catalyzed by ζ-carotene desaturase (ZDS).

In photosynthetically active tissue, lycopene does not accumulate because it serves as an intermediate metabolite in the synthesis of the cyclic and oxygenated carotenoids that act as photoprotectants and light receptors in chloroplast thylakoids. One of the major bicyclic products of lycopene is β-carotene. This compound is the second most abundant carotenoid in ripe fruits because of *b lycopene cyclase*—a specific lycopene cyclase that is expressed exclusively in the chromoplasts of flowers and fruits (Ronen et al., 2000). With *b* lycopene cyclase as an exception, the activity of all lycopene cyclases is greatly reduced during fruit ripening (Fraser et al., 1994; Ronen et al., 1999); this, in concert with increased expression of *DXS*, *PSY1*, and *PDS* genes (Ronen et al., 1999; Lois et al., 2000), allows lycopene to accumulate.

13.6 Phytoene synthase

PSY catalyzes the condensation of two molecules of GGPP (C_{20}) to form phytoene: the basic C_{40} skeleton for the synthesis of all carotenoids (Cunningham and Gantt, 1998).

Tomatoes have two isoforms of PSY that are encoded by differentially expressed *PSY* genes (Bartley and Scolnik, 1993). The accumulation of *PSY1* transcripts occurs primarily in flowers and fruits, including green fruit (Bartley and Scolnik, 1993), and is enhanced in ripening (chromoplastic) fruit (Ronen et al., 1999). *PSY2* is constitutively expressed in all tissues, though transcripts mostly accumulate in green (chloroplastic) tissues such as seedlings, leaves, and green fruits (Bartley and Scolnik, 1993; Fraser et al., 1999).

The partial purification of tomato PSY demonstrates its involvement in metabolite channeling (Fraser et al., 2000). Although PSY is functional in a monomeric state, its native and maximal activity is observed when it is associated with a large (at least 200 kDa) complex containing several proteins, including IPP isomerase and GGPP synthase (Fraser et al., 2000). The possibility exists that PSY serves to anchor the metabolic complex to the thylakoid membrane because the hydrophilicity of the earlier enzymes (IPP isomerase and GGPP synthase) and of the substrates (IPP and DMAPP) is greater than that of PSY and its product (phytoene), which is directly deposited into the membrane (Fraser et al., 2000).

Phytoene synthases contain a conserved domain that includes them among the broad category of class 1 isoprenoid biosynthesis enzymes (cd00385.1, Isoprenoid_Biosyn_C1, NCBI). This superfamily includes *trans*-isoprenyl pyrophosphate synthases (cd00867.1, Trans_IPPS, NCBI), which use C_5 isoprene precursors to synthesize longer isoprenoids and class I terpene cyclases (cd00868.1, Terpene_cyclase_C1, NCBI) that cyclize linear isoprenoids such as geranyl-, farnesyl-, or geranylgeranyl pyrophosphate. These elongation and cyclization reactions both form a new carbon–carbon single bond via the interaction of an electron-deficient allylic carbocation with an electron-rich carbon–carbon double bond. As well, all class 1 isoprenoid biosynthesis enzymes have a catalytic site that consists of a large central cavity formed by mostly antiparallel alpha helices with two aspartate-rich regions located on opposite walls. These residues mediate binding of prenyl phosphates via bridging Mg^{2+} ions, inducing proposed conformational changes that close the active site to solvent, stabilizing reactive carbocation intermediates (Liang et al., 2002).

Within the *trans-IPPS* family, PSY belongs to a group of enzymes possessing a conserved domain that catalyzes head-to-head (HH) (1′-1) condensations (cd00683.1, Trans_IPPS_HH, NCBI). The main representative of this domain, squalene synthase (SQS, EC 2.5.1.21), has received considerable attention because it catalyzes the first committed step of sterol biosynthesis in mammals (Tansey and Shechter, 2000). It proceeds via a two-step reaction in which two molecules of farnesyl pyrophosphate (C_{15}) react to form a stable cyclopropylcarbinyl pyrophosphate intermediate (presqualene pyrophosphate), which is then rearranged and reduced by nicotinamide adenine dinucleotide phosphate (NADPH) to form squalene (Tansey and Shechter, 2000). PSY is similar to SQS in that it forms a cyclopropylcarbinyl pyrophosphate intermediate (prephytoene pyrophosphate), but it differs in that the substrate is geranylgeranyl pyrophosphate (C_{20}) and the prephytoene pyrophosphate intermediate is converted to phytoene by a nonreductive rearrangement, thus NADPH is not required. (Dogbo et al., 1988; Chamovitz et al., 1992; von Lintig et al., 1997).

There are 35 Ser, Tyr, or Thr residues dispersed throughout the PSY Trans_IPPS_HH conserved domain that could be potential targets of phosphorylation as a means to regulate PSY activity posttranslation. Many of these sites are directly adjacent to or within a few bases of several key functional motifs. There are two DDXXD-like motifs responsible for the coordination Mg^{2+} ions located at PSY 161-DXXEDD-165 and PSY 287-DXXED-291. The squalene synthase (SQS) Tyr171 (PSY Tyr242) residue is believed to facilitate farnesyl

pyrophosphate (FPP) ionization by providing a proton from the hydroxyl group to assist the separation of the pyrophosphate moiety and to stabilize the incipient carbocation with its π system (Tansey and Shechter, 2000). There are several potential phosphorylation sites directly adjacent to and within a few positions of these motifs. Finally, there are several potential phosphorylation sites within and surrounding the SQS 50-TSRSF-54 (PSY 131-YAKTF-135) motif, which forms a flap involved in creating a hydrophobic pocket to protect the highly reactive carbocation intermediates from being exposed to the solvent (Pandit et al., 2000). Thus, within the PSY Trans_IPPS_HH conserved domain, there a several potential phosphorylation sites, each located within or near potentially important functional domains.

13.7 PSY1 transgenes

Because of its role in carotenoid biosynthesis of ripening fruit, *PSY1* has received much attention. Transgenic tomato plants expressing antisense *PSY1* cDNA (originally called *pTOM5*) have pale-colored flowers and yellow fruit with a 97% reduction in carotenoid levels (Bird et al., 1991). Understandably, this inhibition of *PSY1* mRNA accumulation did not affect leaf carotenoid levels because of the specificity of the knockout to *PSY1* and not *PSY2* (Bramley et al., 1992). Constitutive *PSY1* overexpression in transgenic yellow fruit r,r-mutants (possessing a loss-of-function mutation in the *PSY1* gene) restored lycopene synthesis to ripening fruit (Fray et al., 1995). However, these same plants exhibited unscheduled pigment production in other cell types, and separate insertion events of the same sense *PSY1* construct caused cosuppression in some plants. In cosuppressed plants, immature green fruit, leaves, and flowers displayed inhibited carotenoid production due to reduced mRNA levels of both the introduced transgene and the endogenous gene (presumably *PSY2*). The profound effects of these irregularities in *PSY1* and *PSY2* transcription demonstrate the importance of these genes in the regulation of carotenoid biosynthesis.

13.8 1-Deoxy-D-xylulose-5-phosphate synthase

1-Deoxy-D-xylulose-5-phosphate synthase catalyzes the condensation of hydroxyethyl thiamine (derived by the decarboxylation of pyruvate), with the aldehyde group of GAP, to form DXP (Lange et al., 1998; Rohmer, 1999). Via subsequent enzymatic steps, DXP is converted to the C_5 building blocks of the isoprenoid pathway (IPP and DMAPP) (Charon et al., 2000; Rodríguez-Concepción et al., 2000).

Complementary DNAs encoding DXS have been cloned from several plant sources that include *Arabidopsis* (Mandel et al., 1996; Estévez et al., 2000), peppermint (Lange and Croteau, 1999), pepper (Bouvier et al., 1998), and tomato fruit (Lois et al., 2000). So far, only one *DXS* ortholog has been found in tomato. Its expression is regulated during development and in an organ-specific manner, showing a strong correlation to carotenoid accumulation in tomato. The tomato *DXS* cDNA has an open reading frame of 2,160 bp flanked by a 156-bp 5′-UTR and a 252-bp 3′-UTR. The cDNA encodes a 719-amino acid polypeptide, with a predicted molecular mass of 77.6 kDa. The polypeptide shows a high degree of sequence similarity with other known DXS polypeptides: 96% identity with that from pepper, 83% with that from *Arabidopsis*, 64% with that from peppermint, and 60% with that from *Escherichia coli*. Plant DXS possesses an N-terminal domain containing

plastid-targeting sequences with an abundance of serine and threonine and low numbers of aspartate and glutamate.

13.8.1 Developmental regulation of DXS, PSY1, PSY2, and PDS

DXS transcripts are abundant in young, developing and fully expanded leaves, inflorescences and stems, and are undetectable in roots (Lois et al., 2000). *DXS* transcript levels in young and mature green fruits are similar to those of other photosynthetically active tissues, but are enhanced greatly during fruit ripening. In situ hybridization studies show a clear correlation between the spatial distribution of *DXS* transcripts (localized to the outer pericarp layers) and the distribution of carotenoids (Lois et al., 2000). *PSY1* is involved primarily in orange or red tissues and *PSY2* in green tissues, though both genes seem to be expressed in all tissues (at least in low levels) (Bartley and Scolnik, 1993; Fraser et al., 1994; Fraser et al., 1999). Phytoene desaturase (*PDS*) is also developmentally regulated, but it is not a rate-limiting enzyme. Its transcripts appear to be low in roots, stems, leaves, and immature/green floral parts, and greater accumulation is observed in mature floral stages and ripening fruit (Bartley and Scolnik, 1993; Giuliano et al., 1993).

The induction of *PSY1* expression begins gradually, before the onset of ripening, at the mature green stage (Lois et al., 2000). The strongest *PSY1* induction occurs at the orange stage, with *PSY1* and *PDS* mRNA levels increasing approximately 25-fold and 16-fold, respectively, from the immature green stage to the orange stage (Ronen et al., 1999). Others confirm increases in *PSY1* and *PDS* mRNA levels at the breaker stage, albeit with varying magnitudes (Pecker et al., 1992; Giuliano et al., 1993; Fraser et al., 1994; Corona et al., 1996; Lois et al., 2000). Concurrent with patterns of carotenoid accumulation, *DXS* transcript levels only begin to increase at the orange stage and then decrease as ripening continues (Lois et al., 2000). *DXS* induction matches most closely with the pattern of carotenoid accumulation, as opposed to *PSY1* induction, which begins at the mature green stage. Therefore, Lois et al. (2000) propose that *DXS*, rather than *PSY1*, controls the initiation of carotenoid accumulation during ripening. There does appear to be some mutual regulation of the two enzymes. Experiments with r,r-mutants (that possess a truncated, nonfunctional form of the PSY1 protein) demonstrate that *PSY1* is required for the final downregulation, but not the initial upregulation of ripening-related *DXS* expression (Lois et al., 2000). Interestingly, the addition of the DXS product, 1-deoxy-D-xylulose (dephosphorylated to improve cellular incorporation), appeared to induce a number of ripening-related processes in fruit such as *DXS* and *PSY1* induction, chlorophyll a degradation, and downregulation of *ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) small subunit (rbcS2)* gene expression (Lois et al., 2000).

Patterns of enzyme activity tend to follow the transcripts levels, although DXS activity has not been measured in tomato. Fraser et al. (1994) report decreases in both PSY and PDS activity of approximately 80% between the mature green and breaker stages. These activities stay relatively constant thereafter, with the exception of PSY, which increases approximately fourfold 7 days postbreaker (d.p.b.). A ripening-related reduction in carotenoid catabolism would result from the destruction of the photosynthetic apparatus, thereby eliminating the carotenoid turnover associated with photosynthesis as well as the reduction of abscisic acid biosynthesis, which uses carotenoid precursors (Scolnik, 1987). Fraser et al. (1994) hypothesize that this near-elimination of carotenoid consumption could allow for their

accumulation at the breaker stage, even with its lower levels of synthesis. However, increased PSY activity at the later stages of ripening would certainly account for the bulk of carotenoid accumulation.

13.9 Photomorphogenesis

Light-mediated changes in plant growth and development are called photomorphogenesis. Phytochromes are one of the three classes of photoreceptors, along with cryptochromes and UV-B photoreceptors that are involved in photomorphogenesis. Phytochromes are red light (R)/far-red light (FR) receptors composed of a protein covalently attached to a linear tetrapyrrole chromophore. Phytochromes are synthesized in the biologically inactive, R-absorbing form: Pr ($\lambda_{max} = 660$ nm). Upon exposure to R, Pr is converted to the biologically active, FR-absorbing form: Pfr ($\lambda_{max} = 730$ nm) (Quail, 2002). Exposing Pfr to FR converts the protein back to Pr, and subsequent exposure to R and FR will enable phytochrome conversion between these two forms. Most phytochrome responses occur at low fluence rates of 1–1,000 μmol/m^2 of light, and are commonly characterized by R/FR reversibility and by reciprocity, which is the requirement for a total number of photons, irrespective of the duration of the light exposure (Neff et al., 2000).

13.9.1 Phytochrome gene family in *Arabidopsis* and tomato

Tomatoes have five phytochrome genes (*PHYA*, *PHYB1*, *PHYB2*, *PHYE*, and *PHYF*) that fall into the same four families as in *Arabidopsis*. They also have a conserved photosensory domain and share (88–98%) amino acid sequence identity to their *Arabidopsis* counterparts (Hauser et al., 1997; Alba et al., 2000b). In dark-grown tissue, phyA is the most abundant of the phytochromes; however, exposure to light causes degradation of phyA in the Pfr form, as well as downregulation of *PHYA* gene expression. Consequently, phyA levels can drop up to 100-fold from light exposure. In light-grown*Arabidopsis* plants, phyB becomes the most abundant isoform, with lower levels of phyC–E (Clack et al., 1994; Hirschfeld et al., 1998; Neff et al., 2000); nevertheless, all five phytochromes are expressed throughout the plant with only minor differences in their expression patterns (Somers and Quail, 1995; Goosey et al., 1997; Neff et al., 2000).

13.9.2 Tomato phytochrome mutants *hp-1* and *hp-2*

A number of tomato mutants exhibit exaggerated photoresponses such as high pigment (hp-1 and hp-2), atroviolacea (atv), and intense pigmentation (Ip) (Kendrick et al., 1994). *hp-1* was found in 1916 at the New Jersey farm of Campbell Soup Co. (Thompson, 1955; Reynard, 1956). A second gene, *hp-2*, was described by Soressi (1975). The two *hp* genes are nonallelic, and a recessive mutant at either locus produces tomato plants with similar characteristics. Mature fruits exhibit high levels of carotenoids, particularly lycopene, and increased vitamin C. The leaves and immature fruit are dark green because of high chlorophyll levels. When grown under continuous R or yellow light, seedlings have shorter hypocotyls and higher anthocyanin levels (Kerckhoffs et al., 1997). Phenotypically stronger alleles *hp-1w* and *hp-2j* have been described (Peters et al., 1989; Van Tuinen et al., 1997).

The tomato *hp-2* gene encodes a homolog of an *Arabidopsis* phytochrome signal transduction gene *DEETIOLATED1* (Mustilli et al., 1999). Since *hp-1* and *hp-2* mutants exhibit many exaggerated light responses (Mustilli et al., 1999), their wild-type gene products appear to inhibit phytochrome signal transduction at a downstream location that is common to both phyA and phyB1 (Kerckhoffs et al., 1997). The fact that increased lycopene accumulation is one of the responses of these mutants suggests that carotenogenesis is mediated by phytochrome.

13.9.3 Phytochromes and tomato fruit

Fruit-localized phytochromes have been found to regulate the extent of lycopene accumulation in tomato fruit; however, they are not required for the initiation of ripening since it will occur in total darkness (Alba et al., 2000a). Ethylene, on the other hand, is necessary for ripening and appears to be the initiating factor (Edwards et al., 1983; Theologis et al., 1993; Bleecker and Kende, 2000). Phytochrome control of lycopene accumulation is not mediated through ethylene, because phytochromes alter neither the timing nor the characteristics of the ethylene burst (Alba et al., 2000a). In addition, phytochrome does not regulate other ethylene-mediated aspects of fruit ripening such as fruit softening and the concentrations of citrate, malate, fructose, glucose, and sucrose (Alba et al., 2000a). In the fruit, *PHYA* transcripts are in greater abundance than transcripts from all other *PHYs* (Hauser et al., 1997), and of all the five *PHY* loci, only *PHYA* showed substantial differential expression during ripening (Alba et al., 2000a). The increase in *PHYA* mRNA accumulation was concurrent with lycopene accumulation. *PHYA* mRNA accumulation was first observed at the breaker stage and increased 11-fold during ripening. It is not known if the increased *PHYA* mRNA led to an equivalent increase in functional phyA photoreceptors. Pigment accumulation in phyA$^-$ mutants does not respond to R or R/FR treatments (Alba et al., 2000a). However, there is still no conclusive evidence that PHYA is the phytochrome responsible for the response. Hauser et al. (1997) reported that *PHYB2* and *PHYF* were preferentially expressed in tomato fruit compared with a variety of organs. This raises the possibility that several *PHY* loci may be involved in regulating carotenoid synthesis (Alba et al., 2000a). Alba et al. (2000a) express interest in using *PHYA*, *B*1, and *B*2 mutants to determine the roles of specific PHYs in tomato ripening.

13.9.4 Mechanism for phytochrome control of carotenogenesis

Phytochrome may regulate carotenogenesis and lycopene accumulation through a number of mechanisms. *DXS* has demonstrated light regulation in *Arabidopsis thaliana* seedlings (Mandel et al., 1996), and *PSY* has demonstrated phytochrome regulation in seedlings of white mustard (*Sinapis alba*) and *A. thaliana* (von Lintig et al., 1997; Welsh et al., 2000). As well, PSY has shown protein activation by light-induced changes to chloroplast membrane composition (Schledz et al., 1996; Welsh et al., 2000).

Recent studies demonstrate the reversibility of PSY activity under red light and far-red light, thus providing the strongest evidence for the phytochrome control of carotenoid accumulation (Schofield and Paliyath, 2005). During in vivo studies, pericarp disks from breaker stage tomatoes were ripened in darkness (D), or D interrupted by daily pulses of red light (R), or R followed by far-red light (FR). After 14-day incubation, R-treated disks

had accumulated 12 mg carotenoids/100 g fresh weight; nearly a 50% increase over D- and R/FR-treated disks. This R/FR reversibility of carotenoid accumulation was also observed in PSY activity 8 days postbreaker, where it showed peak activity. Tomato fruits were also ripened under R, R/FR, and D conditions. *DXS* and *PSY1* transcription was monitored using relative reverse transcriptase-polymerase chain reaction **(**RT-PCR), and PSY activity was estimated using enzyme preparations. However, the R/FR regulation of PSY activity was not reflected in *PSY1* transcript levels. It is not surprising that phytochrome regulation of carotenogenesis occurs at the level of PSY, since it is a branch point from the isoprenoid pathway and is the first committed step of carotenoid biosynthesis. As well, PSY is an important control point for the developmental regulation of carotenogenesis in tomato fruit, with*PSY1* transcript levels and PSY activity increasing during ripening. PSY being a key enzyme of the pathway, any modulation in its activity is likely to be reflected in the levels of other downstream products such as lycopene and carotene.

Recent work with phytochrome signal transduction mutant *high pigment-1* (Cookson et al., 2003) revealed similar results. This mutant showed enhanced phytochrome responses and had higher PSY activity in the ripe fruit, but *PSY1* transcript levels were unaffected. This was somewhat unexpected since it is generally accepted that the control of carotenogenesis during ripening is at the transcript level (Cunningham and Gantt, 1998). Certainly from a developmental perspective, carotenoid accumulation was preceded by increased *DXS* and *PSY1* expression, concomitant with increased PSY activity. One possible explanation is that during development, carotenoid biosynthesis is coarsely regulated by factors such as fruit maturity and the ethylene, and respiratory climacterics, which initiate gene transcription. The fine regulation of this pathway is achieved through other mechanisms, including those mediated by phytochrome. It would seem that these phytochrome effects take place in the form of translational or posttranslational modification of the PSY protein.

A second effect of light has been attributed in part to phytochrome action via *cis*-acting elements in the promoter region of *A. thaliana PSY* (Welsh et al., 2003). Continuous light of all qualities, including FR, R, B (blue), and W (white), increases *PSY* mRNAs (von Lintig et al., 1997) and PSY protein abundance in "membrane pellets" (Welsh et al., 2000). Two tandem ATCTA motifs (−856 to −825) comprise a *cis*-acting element responsible for basal promoter activity in all light conditions (D, W, FR, R, B) as well as enhanced activity under W, FR, R, and B light. This novel ATCTA motif is found in promoter regions of several genes in carotenoid biosynthetic pathway, including *PDS* (*Arabidopsis* and *Zea mays*) and *DXS* (*Arabidopsis*)—which may explain the positive regulation of *DXS* by light (Mandel et al., 1996). A second *cis*-acting element (−210 to −179) containing two short G-box-like motifs is responsible for the differential response toward different light qualities. The G1 motif (CACGAG) is responsible for R light responses (but it also enhances expression in response to W, FR, B), and the G2 motif (CTCGAG) is responsible for W, FR, and B light responses. The promoter region of *PSY1* has not been characterized to explore the existence of any such elements, however, because of the lack of a transcriptional regulation, it appears likely that such light-response elements may not be present for *PSY1*.

13.10 Mevalonate pathway of isoprenoid biosynthesis

A feature, common to all isoprenoids, is their biosynthesis from IPP, the central metabolite and building block for all isoprenoid compounds. Earlier studies were focused on a single

pathway that resulted in the formation of the C_5 monomer, isopentenyl pyrophosphate. In this classic isoprenoid pathway, the precursor of isoprenoids, mevalonate, is synthesized from and 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA), which in turn is formed from acetoacetyl-CoA by the condensation of three acetyl-CoA units. HMG-CoA is converted to the C_6 mevalonate in an irreversible reaction catalyzed by HMG-CoA reductase (HMGR; EC 1.1.1.34). This enzyme catalyzes two reduction steps, each requiring NADPH. Mevalonate is sequentially phosphorylated by two separate soluble kinases, mevalonate kinase and phosphomevalonate kinase, to form 5-pyrophosphomevalonate. Formation of the "active C_5 isoprene unit," isopentenyl pyrophosphate, is then catalyzed by pyrophosphomevalonate decarboxylase (McGarvey and Croteau, 1995).

IPP, along with its isomerization product DMAPP, represents the "activated" monomer building blocks for all isoprenoids. The first isomerization enzyme, IPP isomerase, requires a divalent metal ion and operates through an unusual mechanism involving a carbocation intermediate (McGarvey and Croteau, 1995). Isoprene, the simplest of the isoprenoids, is synthesized directly from DMAPP by the enzyme isoprene synthase, eliminating the diphosphate unit. Condensation of DMAPP with IPP in a head/tail fashion by various prenyltransferases generates prenyl diphosphates of different chain lengths. The C_{10} compound, geranyl pyrophosphate (GPP), is catalyzed by GPP synthase. Addition of a second IPP unit to GPP generates the C_{15} compound FPP by FPP synthase; and addition of a third IPP generates GGPP by GGPP synthase; and so on (McGarvey and Croteau, 1995). The families of enzymes responsible for the conversion of GPP, FPP, and GGPP to the monoterpene, sesquiterpene, and diterpene classes, respectively, are referred to as monoterpene, sesquiterpene, and diterpene synthases or cyclases, and represent reactions committing carbon from the central isoprenoid pathway to the end products (Chappell, 1995).

In higher plants, at least three distinct semiautonomous subcellular compartments exist that synthesize isoprenoids: cytoplasm/ER (sesquiterpenes and triterpenes, e.g., sterol), plastids (monoterpenes and diterpenes, e.g., chlorophyll, carotenoids, and prenylquinones), and mitochondria (ubiquinones). It is generally accepted that at least the final biosynthetic steps are bound to these compartments. The biosynthesis of particular mono- and diterpenes is generally attributed to the plastidic compartment, even if other subsequent biosynthetic steps and accumulation of the final isoprenoid may occur in separate compartments (Lichtenthaler et al., 1997).

13.11 HMG-CoA reductase as the key regulatory enzyme

HMGR, a highly conserved enzyme in eukaryotes, catalyzes the rate-limiting step of IPP biosynthesis in animals and most of the isoprenoid biosynthesis in plants. In higher plants, HMGR is encoded by a multigene family (Lichtenthaler et al., 1997) with the genes characteristically distinguishable from each other by the sequence differences at the 3′-untranslated regions of the cDNAs (McCaskill and Croteau, 1997). As well, *HMGR* genes are nuclear-encoded (Lichtenthaler et al., 1997). HMGR is encoded by at least two distinctive genes in *A. thaliana* (Caelles et al., 1989), cotton (*Gossypium hirsutum* L.) (Loguercio et al., 1999), and rice (*Oryza sativa*) (Nelson et al., 1994); by three genes in rubber (*Hevea brasiliensis*) (Chye et al., 1992), tomato (*Lycopersicon esculentum*) (Weissenborn et al., 1995), and potato (*Solanum tuberosum*) (Yang et al., 1991; Choi et al., 1992); and an even larger

complex multiple gene family in maize (*Z. mays*) and pea (*Pisum sativum*) (Weissenborn et al., 1995).

The presence of multiple genes is consistent with the hypothesis that different isoforms of HMGR are involved in separate subcellular pathways to produce specific isoprenoid end products (Stermer et al., 1994; Rodríguez-Concepción and Gruissem, 1999). Plant HMGR activity responds in vivo to a variety of developmental and environmental signals, such as cell division, light, and pathogen infection (Stermer et al., 1994). Plants regulate HMGR activity at the level of mRNA by differential induction of *HMGR* gene family members, and posttranslationally by enzyme modification (Stermer et al., 1994) by a protein kinase cascade in which phosphorylation inactivates the enzyme (McCaskill and Croteau, 1997). Calcium, calmodulin, and proteolytic degradation may also have a role in regulation of plant HMGR (Stermer et al., 1994).

It has been suggested that some of the HMGR isoforms may be involved in separate subcellular pathways for specific isoprenoid biosynthesis through metabolic channels, or "metabolons" (Stermer et al., 1994). A number of investigators have reported a correlation between the induction of isoprenoid biosynthesis, particularly that of sesquiterpenes, and HMGR enzyme activity (Chappell et al., 1995). In potato, the expression of specific *HMGR* genes has been correlated with the accumulation of steroids or sesquiterpenes (Choi et al., 1992). Chye et al. (1992) observed that only *hmg1* was inducible by C_2H_4 among other *HMGR* genes, and also speculated that distinct isoprenoid pathways do occur for rubber biosynthesis in *H. brasiliensis*. In cotton, *hmg2* has been associated with the synthesis of specific sesquiterpenes in developing embryos (Loguercio et al., 1999). These results also support the concept of metabolic channels, or arrays of isoenzymes, independently regulated and specifically dedicated to the production of particular isoprenoids (Chappell, 1995).

HMGR isoforms are expressed differentially in response to a variety of developmental and environmental stimuli such as fruit development, phytohormone levels, endogenous protein factors, light, and pathogen infection (Stermer et al., 1994). In tomato, *hmg1* is highly expressed during early stages of fruit development, when sterol biosynthesis is required for membrane biogenesis during cell division and expansion (Narita and Gruissem, 1989), whereas *hmg2* expression, not detectable in young fruit, is activated during fruit maturation and ripening (Rodríguez-Concepción and Gruissem, 1999). Cotton *hmg2* encodes the largest of all plant HMGR enzymes described to date, and contains several functional specialization features that include a unique 42-amino acid sequence located in the region separating the amino-terminal domain and carboxy-terminal catalytic domain, which is absent in *hmg1* (Loguercio et al., 1999).

13.12 Regulation of α-farnesene biosynthesis in apples

In addition to several ester volatiles that impart the characteristic aroma, apple fruits also produce large amounts of the acyclic sesquiterpene α-farnesene ($C_{15}H_{24}$; [3*E*,6*E*]-3,7,11-trimethyl-1,3,6,10-dodecatetraene), which accumulates in the skin of apple fruit after harvest during low-temperature (0–1°C) storage (Paliyath et al., 1997; Rupasinghe et al., 2000a). The extent of oxidation of α-farnesene to conjugated trienes has been shown to be proportional to the development and severity of the postharvest physiological disorder superficial scald (Huelin and Coggiola, 1970). Biosynthesis of α-farnesene in apple skin is highly regulated by temperature (Rupasinghe et al., 2000a) and C_2H_4 (Gong and Tian,

1998; Rupasinghe et al., 2000b, c). Recently, as a first step toward understanding the regulation of α-farnesene accumulation by HMGR, total in vitro HMGR enzyme activity and expression of two novel cDNA clones, *hmg1* and *hmg2*, encoding HMGR were studied in the skin tissue of apple in relation to low-temperature storage and C_2H_4 action.

Accumulation of the stress metabolite α-farnesene in the skin of apples is triggered by low-temperature storage and reaches a peak during 4–12 weeks in storage (Rupasinghe et al., 2000a, b). In contrast, in vitro HMGR activity was the highest at the time of harvest and gradually decreased during the first 8 weeks of storage, and then remained constant during the remainder of the storage period. Incorporation of radiolabeled or unlabeled mevalonic acid into α-farnesene is favored over a mixture of GAP and pyruvic acid (precursors of Rohmer pathway) in isolated apple skin tissues. Therefore, it is evident that the biosynthesis of α-farnesene occurs predominantly through the classical mevalonate (MVA) pathway in apple fruit. This conclusion is supported also by the observation that lovastatin, a competitive inhibitor of HMGR, inhibits α-farnesene accumulation significantly (by 25–54%) in apple skin during storage. Recently, Ju and Curry (2000) also found that when lovastatin is applied to apple fruit tissue at high concentrations, α-farnesene biosynthesis is suppressed to undetectable levels in "Delicious" and "Granny Smith" apples. Together these results imply that in apple fruit the biosynthesis of α-farnesene occurs predominantly through the classical MVA pathway.

Cloning and expression of an α-farnesene synthase cDNA from apple ("Law Rome") peel tissue was achieved by Pechous and Whitaker (2004). Using degenerate primers, an 800-bp cDNA fragment of α-farnesene synthase was isolated. A cDNA library generated from peel tissue mRNA was screened to isolate a 1,931-bp long full length terpene synthase cDNA (AFS1; GenBank accession number AY182241), which contained an open reading frame of 1,728 bp encoding a protein containing 576 amino acids and having a relative molecular mass of 66 kDa. The sequence had characteristics similar to monoterpene synthases. The protein was functionally expressed in *E. coli* and converted farnesyl pyrophosphate into (E,E)-α-farnesene. Northern blots showed that the AFS1 transcript levels increased fourfold in apple peel tissue during storage for a period of 4 weeks at 0.5°C. There appears to be a better correlation between an increase in α-farnesene synthase activity and α-farnesene synthase transcript levels than HMGR activity. The expression of *AFS1* is also ethylene-dependent since treatment with 1-methylcyclopropene (1-MCP), an ethylene receptor blocker, reduced the AFS1 transcript levels during the initial 4-week period of storage, with further inhibition to undetectable levels during extended storage up to 8 weeks. α-Farnesene levels were also reduced in apples by treatment with aminoethoxyvinylglycine and 1-MCP, further supporting the role of ethylene in farnesene biosynthesis (Rupasinghe et al., 2000c).

13.13 Isolation of *hmg1* and *hmg2* cDNA from apple and their expression during storage

To further study the regulation of HMGR activity in relation to the accumulation of α-farnesene in apple fruit, a molecular approach was employed. With these objectives, a full length (*hmg1*) and a fragment (*hmg2*) of cDNAs of two *HMGR* genes from the skin of apple fruit were cloned using a strategy, utilizing sequence similarities among previously cloned plant *HMGR* genes (Rupasinghe et al., 2001). All plant *HMGR* genes identified to date share some common structural features (Maldonado-Mendoza et al., 1997). They are highly

conserved in the carboxyl terminal region, highly divergent in the amino terminal region, and possess two putative transmembrane domains. The presence of two transmembrane domains in the amino terminal region is a common feature of all the *HMGR* genes cloned to date from plants, but differ from the animal *HMGR* gene that has seven transmembrane domains (Liscum et al., 1985). Northern blot analysis revealed that *hmg1* and *hmg2* genes were expressed differentially in apples during cold storage. It is interesting to note that *hmg1* was constitutively expressed throughout the 16-week postharvest storage period. The transcripts for *hmg2* showed the highest levels in parallel with the accumulation of α-farnesene in the skin, which also increased during storage. The increase in abundance of *hmg2* transcript coincides with endogenous C_2H_4 production in apples during storage. However, the abundance of *hmg2* mRNA was relatively low compared with that of *hmg1*. The differential regulation of *hmg1* and *hmg2* expression in apple is consistent with the theory that levels of the different HMGR isozymes in plants are modulated in response to specific developmental and stress signals (Stermer et al., 1994). The expression of *hmg2* resembled the in vitro changes of HMGR activity during storage, more than that of *hmg1*. The poor correlation between total HMGR activity and farnesene accumulation could be due to several factors such as posttranscriptional events such as mRNA processing, transcript stability, nucleocytoplasmic transport, translation efficiency, and/or protein modification and half-life. HMGR is regulated by a protein kinase cascade in which phosphorylation inactivates the enzyme (McCaskill and Croteau, 1997).

13.14 Regulation of HMGR by ethylene

C_2H_4-mediated stimulation of α-farnesene biosynthesis is partly due to the induction of HMGR activity by C_2H_4 (Rupasinghe et al., 2001). In apples, the C_2H_4 action inhibitor 1-MCP suppressed the expression of *hmg2* completely and *hmg1* partially. 1-MCP inhibited respiratory CO_2 evolution by 50%, suggesting that inhibition of α-farnesene synthesis in apple by 1-MCP could be also regulated through the available acetyl CoA pool that is utilized by isoprenoid pathway (Rupasinghe et al., 2001). However, it is clear from the literature that C_2H_4 or other stimuli, which induce C_2H_4 production, can influence differential expression of the isogenes of HMGR. In rubber, *hmg1* is induced by C_2H_4, while *hmg3* expression remains stable, indicative of a housekeeping nature of the gene (Chye et al., 1992). Furthermore, *hmg1* is expressed predominantly in the laticifers, the cells specific to rubber biosynthesis (Chye et al., 1992); thus, it is postulated that *hmg1* of rubber encodes the HMGR enzyme involved in rubber biosynthesis. In tomato, *hmg1* expression is very high at early stages of fruit development but declines during ripening (Narita and Gruissem, 1989), but *hmg2* is highly expressed during ripening (Rodríguez-Concepción and Gruissem, 1999). In *Camptotheca acuminata*, *hmg1* mRNA increased in response to wounding, but *hmg2* and *hmg3* transcript levels remained unaffected (Maldonado-Mendoza et al., 1997). In potato, *hmg2* mRNA levels are elevated in response to wounding or fungal elicitors suggesting that *hmg2* is a defense-related gene or *hmg2* is the major elicitor-induced isogene (Yang et al., 1991).

Plants regulate HMGR activity at the level of mRNA by differential induction of *HMGR* gene family members, and posttranslationally by enzyme modification (Stermer et al., 1994). In *C. acuminata* apices, *hmg1* is expressed at high levels, *hmg3* is moderately expressed, and *hmg2* transcripts are absent (Maldonado-Mendoza et al., 1997). It is speculated that the

expression of specific *HMGR* genes in specific organs and tissues in the plant could be used as a mechanism for regulating the supply of mevalonate to metabolic pathways localized in those places.

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Chapter 14

Postharvest Treatments Affecting Sensory Quality of Fresh and Fresh-Cut Products

Elazar Fallik

14.1 Introduction

Consumer interest worldwide in the quality of fresh and fresh-cut products has increased in recent years. Product quality is a complex issue, since it includes visual characteristics, physical properties such as texture, mineral and vitamin content, flavor, and other organoleptic characteristics. Fruits and vegetables are also appreciated for their beneficial health effects in humans (so-called nutraceutical properties). Once produce is harvested, postharvest handling practices do not usually improve on the quality attained in the field; they only slow the rate at which deterioration occurs. Appearance, flavor, and freshness of a product can play a principal role in consumer's decision to purchase it and can influence perception by other senses (Peneau et al., 2006). Flavor is composed of two components: taste and odor. Taste is classified into five basic sensations: sweet, sour, salty, bitter, and umami (in Asia/Japan). Odors are created by volatile compounds that are perceived by the nose, directly or retronasal. Total flavor perception is thus a function of the composition of taste and odor compounds, their interaction with receptors in the nose and tongue, and recognition by the brain. However, flavor and aroma depend on many factors. The large genetic variability in nature results in differences in flavor among cultivars. In addition, maturity and postharvest handling significantly affect the sensory quality of fresh produce (Forney et al., 2000).

Perceptions of what constitutes good quality vary between countries, regions, and individuals. They can also be affected by culture, experience, and personal preferences. The overall quality attributes that are important to packers, transporters, and retailers are often quite different than those of consumers (Watkins and Ekman, 2005). Although the trade ensures that consumers are presented with products of excellent appearance and at least acceptable texture, flavor is often ranked lower in importance by marketers (Watkins and Ekman, 2005). However, sensory quality is important to consumer satisfaction and influences further consumption.

Many consumers are dissatisfied with the flavor and quality of fresh or fresh-cut products (Bruhn, 1995; Baldwin, 2002), even though they have access to a greater variety of produce than ever before. Good postharvest technology helps to maintain produce quality. However, there is no general agreement about what quality is, how it can be measured, and how

it relates to consumer acceptability (Shewfelt, 1999). Quality has been defined as "the composite of those characteristics that differentiate individual units of a product, and have significance in determining the degree of acceptability of that unit by the buyer" (Shewfelt, 1991). The key to increasing consumer consumption of fresh fruits and vegetables and fresh-cut products, without loss of grower income, lies in providing produce with superior flavor that lasts during an extended shelf life.

This chapter summarizes recent progress on postharvest (and some preharvest) methods that affect sensory quality of freshly harvested and fresh-cut products.

14.2 Preharvest treatments

The ultimate potential postharvest quality and shelf life of fresh vegetables is determined before harvest. Several variables that include the cultivar, weather conditions, irrigation practices, fertilizer, and pest control programs affect the harvested quality of produce and its sensory properties. Indoor production of fresh vegetables helps assure uniform crop quality compared to outdoor production, because the plants are not exposed directly to rapid changes in climate. On the other hand, vegetable cultivation in a greenhouse under artificial conditions can also affect the organoleptic quality of the product. This is reflected in different flavors compared with those found in field-grown vegetables (Gruda, 2005). The soil type used for cantaloupe (*Cucumis melo*) production, as well as storage duration, can affect postharvest sensory attributes of the fruit. Melons grown in sandy loam were lower in sweet aromatic and sweet taste and higher in moisture release and fermented flavor than the fruits grown in heavy clay soil. Fruity/melon, sweet aromatic, surface wetness, hardness and moisture release attributes decreased while fermented and sour flavor increased during storage, regardless of soil type. Clay soil appeared to have some advantages over sandy loam soil in producing cantaloupe fruits with better sensory quality attributes (Bett-Garber et al., 2005).

Fertigation is also a very important preharvest aspect that affects quality and sensory attributes of fresh produce. In a recent study, strawberries were exposed to three different levels of salinity. Fruit quality, characterized as taste, aroma, and texture by a consumer panel, decreased by more than 24% with increasing salinity in one cultivar, but was unaffected in a different cultivar (Saied et al., 2005). Neither sensory quality attributes nor the composition of volatile compounds were affected by the planting density of apples (*Malus* × *domestica* Borkh.) cultivars (Thybo et al., 2005) or canopy density of kiwifruit (*Actinidia deliciosa* "Hayward") (Snelgar et al., 1998). However, the variation in some sensory flavor attributes between apple cultivars was correlated with the concentrations of volatile compounds, of which the majority were straight-chain and branched-chain esters of fatty acids with characteristic fruity and/or sweet notes (Thybo et al., 2005). In a different study, the quality of apple fruits was evaluated under organic (ORG), conventional (CON), and integrated (INT) growing conditions. Soil classification, rootstock, cultivar, plant age, and all other conditions except management were the same on all plots. Fruit volatile production and sensory panels were used to evaluate fruit quality. Consumer panels rated ORG and INT apples to have equal or better overall acceptability than CON apples, partially because of higher aroma volatiles (Peck et al., 2006).

Virtually, some of the postharvest quality factors are genetically controlled and can vary with variety. Therefore, from a quality standpoint, cultivar selection may be an important

management decision in crop production (Hampson and McKenzie, 2006). Cultivar and planting site are two factors that often receive minimal attention, but can have a significant impact on the quality and flavor of many fruits and vegetables (Barritt, 2001). Miller et al. (2005a) reported that apple fruit quality and sensory variables measured were affected in 20 different cultivars. Similar findings have been reported in a study conducted in 13 apple varieties (Young et al., 2004). Principal component analysis clustered the apples into three groups according to skin color: red, green, and red green. Total ester contents were highest with the red apples, and the green apples had the highest α-farnesene levels, thus affecting the sensory attributes of the fruits (Young et al., 2004). Flavor was affected by the cultivar $\times$ planting site interaction. Attractiveness and desirability were two other sensory attributes that were affected by the apple cultivar $\times$ planting site interaction (Miller et al., 2005b). The sensory evaluations of sweetness, taste, and aroma were well correlated with chemical attributes of peach and nectarine quality in nine cultivars (Colaric et al., 2005). Cultivar was more important than the harvest date in aroma volatile production by strawberries (Pelayo-Zaldivar et al., 2005). Similar conclusions were reported for blackberries over three growing seasons (Qian and Wang, 2005). Despite seasonal variations, cultivar variations contributed more to the total amount of volatiles and therefore to the sensory attributes. Three tomato cultivars differed in their contents of aroma flavor compounds and their intensity of sensory attributes. Changes in most of the flavor compounds after harvest phase were similar, despite the different levels of the aroma compounds in the different cultivars at harvest. In all three cultivars, the intensity of the attribute "tomatolike" (odor, flavor, and aftertaste) increased, but so did the intensity of the undesired attribute "moldy" (odor, flavor, and aftertaste). Changes of flavor components and related sensory attributes appeared to lead to an altered balance of potentially desirable and undesirable properties when tomatoes were stored ripe (Krumbein et al., 2004). In a different study conducted on different tomato cultivars, significant differences among very closely related tomato cultivars could be detected for volatile aromas and sensory testes (Ruiz et al., 2005). The texture and the sensory quality and acceptability of melon cultivars seem to be linked with the genotype, which can be differentiated on the basis of retronasal odor (Hoberg et al., 2003).

The effects of preharvest treatments on apple quality and sensory were studied over three seasons using "Delicious" apple trees. The bioregulators aminoethoxyvinylglycine (AVG) and ethephon (ETH) were applied alone or in combinations at various time intervals before harvest. Fruit response to bioregulators was evaluated at harvest and after storage. AVG in all instances reduced the sensory scores for apples and apple juice. Sensory values for whole apples were not influenced by ETH treatment, but ETH improved sensory preference for apple taste, particularly at early harvest. Applying AVG before ETH enhanced soluble solids and sensory scores for the fruit. Fruits treated with AVG followed by ETH had better quality and sensory perceptions (Drake et al., 2005).

14.3 Harvest and stage of maturity

Stage of maturity at harvest is a very important factor in determining the quality of the fresh harvested commodities. Immature fruits are more subject to shriveling, internal breakdown, mechanical damage, and inferior quality when they finally ripen after harvest. Overmature fruits are likely to become soft and mealy, and attain insipid flavor soon after harvest.

"Blackamber" plum (*Prunus salicina* Lindell) consumer acceptance and market life were highly dependent on harvest date. Based on this work, "Blackamber" plums were well adapted to late harvest, but proper postharvest temperature management, including ripening, and marketing within its market life potential are necessary to maintain flavor (Crisosto et al., 2004). The influence of maturation and storage on quality of plum (*Prunus domestica* L.) cultivars was evaluated after harvesting from early to overripe ripening stages (Kreck et al., 2005). There was a large acceptance of fresh overripe fruits picked at the end of the harvesting period. Although statistical correlations between analytical and sensorial parameters were mainly found between sweetness and soluble dry mass and between the attributes sourness/unripe/green/astringency and total acidity, sweetness and sourness were the deciding factors in determining the general acceptability of the fruits by consumers. Apple aroma volatiles and sensory quality were significantly affected by harvest date based on the fruit color. Higher red color fruit had higher concentrations of aroma volatiles than the lower red color grading (Thybo et al., 2005). The quality parameters of muskmelon (*C. melo* L var. *reticulatus* Naud) are often related to sugar content and aroma composition. The aroma development and sensory assessment in two Israeli "Galia" melon cultivars (*C. melo* var. *reticulatus*, cv. C8 and cv. 5080) was monitored, based on two ripening stages (partially ripe and ripe). The best quality and sensory attributes were obtained with fruit that was harvested when light yellow with some green areas. At this stage of maturity, fruit had a longer shelf life and a better aroma and sensory qualities (Fallik et al., 2001). Similar results were reported by Senesi et al. (2005) for two muskmelon varieties that were assayed for quality and sensory at three different ripening stages (unripe, ripe, and overripe) and by Lalel et al. (2003) with mango. The pulp of ripe mango fruit harvested at the sprung green stage exhibited higher total amounts of aroma volatiles than fruit harvested at either earlier (mature green) or later (half-ripe or ripe) stages. Harvest date was found to affect sensory quality of carrots. Delaying the harvest date increased the sensory scores for crispness, sweetness, and overall flavor and decreased scores for bitterness. Storage, on the other hand, had little effect on sensory quality (Suojala and Tupasela, 1999).

14.4 Postharvest treatments

Postharvest handling practices do not improve the quality of fruit after harvest; they only slow the rate at which deterioration occurs. Practices such as washing, sorting, sizing, packing, wrapping, and temperature management are services for the consumer, and generally do not improve inherent quality.

14.4.1 Controlled atmosphere

Controlled atmosphere (CA) storage is used to extend the storage life of seasonal perishables. CA refers to the introduction of low oxygen (O_2) and/or high carbon dioxide (CO_2) atmosphere to a container or an airtight enclosure holding the product. The atmosphere is "controlled" by a sequence of measurements and corrections throughout the storage period and is used as a supplement to proper refrigerated storage and distribution. Controlled atmosphere has been used historically in large storage facilities where levels of atmospheric gases are continuously monitored and adjusted to maintain optimal concentrations. It is typically applied to commodities amenable to long-term storage such as apples, pears, or

kiwifruit. More recently, controlled atmospheres have been introduced to refrigerated containers for long distance transport by sea. The technology is being used for stone fruits and other commodities. The use of CA is increasing due to improved cost-effectiveness in delivering extended shelf life and enhanced produce quality. However, this technology may affect the volatile composition of the room atmosphere, which in turn may affect the volatile production of ripe fruit.

"Fuji" apples (*Malus* × *domestica* Borkh.) were harvested at two different dates, over two consecutive years, and stored under different atmosphere conditions (CA and ultra-low oxygen). Generally, the highest total aroma emission was obtained after 5-month storage and 1 day of incubation at 20°C regardless of atmosphere conditions, for early-harvested fruit. After 7-month storage, the ultra-low oxygen atmosphere depressed total aroma volatile emission (Echeverria et al., 2004a, b). Physicochemical parameters of "Fuji" apples were preserved throughout storage, especially in CA-stored apples; however, these apples showed lower total aroma emission. Sensory acceptability was also higher for CA-stored fruit after 7 months of storage, whereas no significant differences were found for shorter storage periods. Varela et al. (2005) reported that consumer acceptability and descriptive sensory analyses of apples, for storage periods of up to 28 days at 20°C, indicated that the greatest quality loss was associated with increased alcoholic taste and odor. Treating "Gala" apples with 1-MCP (1-methylcyclopropene) and then storing them in air or CA or storage in CA without 1-MCP treatment reduced volatile production compared to apples not treated with 1-MCP and stored in air (Mattheis et al., 2005). "Gala" quality, sensory characteristics, and volatile production were also characterized following regular atmosphere (RA) storage without and with a prestorage heat treatment (38°C for 4 days) or CA storage (Saftner et al., 2002). Volatile levels were reduced following CA storage compared with RA storage with or without a prestorage heat treatment. Overall acceptability was higher for CA- than for RA-stored fruit. Sensory scores for sweetness were similar among all treatments. Quality and sensory characteristics were generally similar in heated and nonheated regular atmosphere-stored fruit, and in controlled atmosphere-stored fruit.The results indicate that short-term CA storage can maintain instrumental and sensory quality of "Gala" apples (Saftner et al., 2002). CA was found to affect the sensory quality of other crops. A CA storage comprising 2 kPa O_2 and 3 kPa CO_2 or 3 kPa O_2 in combination with 6 kPa CO_2 at 13°C seems to be promising for extending the shelf life of the "Kensington Pride" green mature mango (*Mangific a indica* L) while still maintaining a high concentration of the major volatile compounds responsible for the aroma of ripe mangoes (Lalel and Singh, 2004; Lalel et al., 2005). CA storage also affected the sensory quality of "Tommy Atkins" mango (Bender et al., 2000). Fruits were harvested at mature green (MG) and tree ripe (TR) stages and stored for 21 days in air or in CA. The results suggested that properly selected atmospheres, which prolong mango shelf life by slowing ripening processes, can allow TR mangoes to be stored or shipped without sacrificing their superior aroma quality. Mahajan and Goswami (2004) reported that sensory evaluation of litchi (*Litchi chinensis* Sonn.) held in CA was rated good throughout 56 days of storage. The volatile profiles of ripe kiwifruit and sensory quality differed between CA-stored and air-stored fruits, and also among fruits from the different CO_2 scrubbing systems. However, it remains to be determined whether the differences are significant to consumers (Burdon et al., 2005). The postharvest life and flavor quality of three strawberry (*Fragaria* × *ananassa* D.) cultivars kept at 5°C in air or at CA (20 kPa CO_2) were investigated. Under CA conditions, flavor quality was maintained longer than

under air (Pelayo et al., 2003). Keeping wild strawberry fruits (*Fragaria vesca* L.) at under 10 kPa of CO_2 and 11 kPa O_2 efficiently prolonged the shelf life by maintaining the quality parameters within acceptable values, without significantly modifying consumer acceptance (Almenar et al., 2006). The flavor and external appearance of grapefruit stored for 21 days at 14°C under ultra-low oxygen and 14 additional days in air at 23°C was rated acceptable, yet inferior to grapefruit stored similarly in air (Shellie, 2002). On the other hand, freshly harvested "Kyoho" table grapes (*Vitis vinifera* × *Vitis labrusca*) were stored in controlled atmosphere, either in high oxygen concentration (80 kPa O_2) or 40 kPa O_2 + 30 kPa CO_2. Storage in 80 kPa O_2 or 40 kPa O_2 + 30 kPa CO_2 improved flavors over control fruits stored in air. Treatment in 80 kPa O_2 resulted in significantly high sensory scores than those treated with 40 kPa O_2 + 30 kPa CO_2, but did not significantly affect the aroma (Deng et al., 2005).

The effect of CA on sensory quality of several vegetables was also tested. Mature green, breaker, and pink tomatoes (*Lycopersicon esculentum* Mill.) were treated in air or in CA at 0.5 kPa O_2 and 80 kPa CO_2. Aroma and taste were not enhanced by any treatment/ripeness combinations, although high CO_2 marginally increased the sweetness and blandness of the fruit (Ratanachinakorn et al., 1997). Sensory assessment of green asparagus (*Asparagus officinali*) indicated that spears held in CA for 6 days had similar flavor and acceptability to spears held in air for 1 day. Spear quality was more strongly influenced by CA than by feeding solutions (Renquist et al., 2005). Gomez and Artes (2004) reported that CA improved sensory quality of green celery stalks. Neither off-odors nor off-flavors were detected in stalks from any treatment.

14.4.2 Modified atmosphere packaging and coatings

Modified atmosphere packaging (MAP) involves the modification of the head space gas in a package in order to prolong the shelf life of the product it contains. The success of MAP depends on the packer's ability to correctly prepare the product and to control the concentrations of head space gas within the desired limits. Edible coatings and films can provide an alternative for extending the postharvest life of fresh fruits and vegetables.

Bagging of peach (*Prunus persica*) on tree increased fruit flavor through an increase in aroma volatile content (Jia et al., 2005). The postharvest quality of papaya (*Carica papaya* L.) was enhanced significantly by combining MAP (3–5 kPa O_2; 6–9 kPa CO_2) with methyl jasmonate treatments. The modified atmosphere created inside the package did not induce any off-flavor development during storage at 10°C (Gonzalez-Aguilar et al., 2003). Harvested fresh mature green papaya (*C. papaya* L.) fruits were individually shrink-wrapped with Cryovac D-955® film. The shrink-wrapped papaya fruits could be stored for 10 days at ambient temperature, after which they ripened normally in 5 days with firm texture and good flavor after unwrapping, whereas the nonwrapped fruits ripened within 7 days beyond which they became unmarketable (Singh and Rao, 2005).

Strawberries (*F. ananassa*) are highly perishable and are characterized by a short shelf life. Wheat gluten-based coatings and films maintained the visual quality of refrigerated strawberries during storage, and the taste was acceptable to consumers. However, the appearance and taste of coated fruit were unacceptable (Tanada-Palmu and Grosso, 2005). Chitosan has been found to be an ideal preservative coating material for fresh strawberries

due to its antifungal and film-forming properties (Han et al., 2005). Three 1% chitosan-based solutions were developed for coating strawberries: chitosan in 0.6% acetic acid solution, in 0.6% lactic acid solution, and in 0.6% lactic acid solution plus 0.2% vitamin E. Trained panel showed that chitosan-coated strawberries have similar sensory descriptors as those of fresh berries, whereas coatings containing vitamin E developed the waxy-and-white surface of the samples. The trained panel did not detect astringency difference among all samples, indicating that 1% chitosan coating did not change astringency of strawberries. A novel edible coating based on aloe vera gel has been used as a means of preservation to maintain the quality of cv. "Crimson" seedless table grapes during cold storage and subsequent shelf life (Valverde et al., 2005). After 7 days at 1°C plus 4 days at 20°C, the sensory analyses of clusters treated with aloe vera gel revealed beneficial effects such as slow weight loss and color changes, without any detrimental effect on taste, aroma, or flavors. Similar results with beneficial effects regarding the sensory quality were reported for sweet cherry treated with aloe vera gel (Martinez-Romero et al., 2006). The sensory traits of "Galia"-type melon fruit coated with three polyethylene-based waxes with different solid matter and shellac contents ("Zivdar," "Tag," and "Tag-A") or with natural beeswax ("Beeswax") was evaluated after prolonged storage. "Tag"-treated melons had better sensory quality, as evaluated by organoleptic tests and aroma volatiles. Treatment with the waxes "Zivdar" and "Tag-A," which contain high amounts of shellac in proportion to "Tag" and "Beeswax," significantly increased off-flavor in the melon fruit due to high internal levels of CO_2, ethanol, acetaldehyde, and ethyl acetate. Untreated fruit, or fruits that were coated with "Beeswax," had the best taste (Fallik et al., 2005b). "Mor" is a new mandarin variety, which has a rich aromatic taste. The variety suffers from the development of off-flavors, which are often enhanced by coating the fruit with waxes, which restrict gas exchange and lead to the development of anaerobic conditions in the internal atmosphere of the fruit. Porat et al. (2005) found that, in order to maintain the best sensory quality of the fruit, it was necessary to include at least 13% of total solids and half the regular amount of shellac to improve fruit taste and reduce the formation of off-flavors, compared with fruit coated with the commercial wax formulation.

14.4.3 Heat treatments

Heat treatments appear to be one of the most promising means for postharvest control of decay (Lurie, 1998). Heat treatments against decay-causing agents may be applied to fruits and vegetables in several ways: by hot water dips, by vapor heat, or by hot dry air (Lurie, 1998), or by a short hot water rinsing and brushing (Fallik, 2004). Heat treatments can also be used to inhibit ripening processes, or enhance resistance to chilling injury during storage, thus extending storability and marketing (Lurie, 1998).

Heat treatment of apple fruits (4 days at 38°C) markedly inhibited emission of total volatile esters, compounds commonly associated with apple aroma of apple within 1 day of treatment. However, after an extended refrigerated storage at 1°C, the heat-treated fruit recovered and produced more total volatiles, compared to non-heat-treated fruit (Fallik et al., 1998). Abbott et al. (2000) found that calcium infiltration significantly increased sensory and overall acceptability scores of "Golden Delicious" apples held at 38°C for 4 days. Apples exposed to heat before storage were sweeter than those not heated. Only the untreated (unheated) fruits were scored in the unacceptable range for crispness and overall

acceptability. Immersing sapote mamey fruit (*Pouteria sapota*) at 40–60°C for 40–60 min did not influence the aroma and taste of the fruit (Diaz-Perez et al., 2001). Hot water-treated ber fruits (*Ziziphus mauritiana* Lamk, cv. "Umran") at 50°C for 5 min and stored in modified atmospheric bags (sealed polythene bags) were qualitatively better than control fruits in terms of taste and appearance, even after day 8 of storage. The control fruits were not found acceptable after day 4 of storage (Lal et al., 2002). Heating Tarocco blood oranges (*Citrus sinensis* Linn. Obsek) at 37°C for 48 h after harvest adversely affected fruit flavor and taste after storage (Schirra et al., 2002). Intermittent warming at 2°C +1 week at 11°C and temperature conditioning for 7 days at 16°C affected pummelo-grapefruit hybrid fruit (*Citrus grandis* × *Citrus paradise* cv. Oroblanco) taste and the amounts of off-flavor volatiles emitted from the fruit. Taste panels indicated that the taste score of untreated control fruit gradually decreased during long-term storage. The taste of intermittent warming-treated fruit remained acceptable even after 16 weeks of storage, and TC-treated fruit remained acceptable for up to 12 weeks (Porat et al., 2003).

14.4.4 Temperature management

Temperature management is one of the most important factors affecting the quality of fresh produce. There is an optimum storage temperature for all products. The ideal temperature often depends on the geographic origin of the product. Temperature management is a key tool to extend storability and shelf life of the fresh harvested produce, by slowing both physiological and pathological deterioration.

The production of aroma compounds of strawberry fruit (*Fragaria* × *ananassa* ev. Chandler) was markedly influenced by storage time and temperature. Strawberries stored at 0°C retained an acceptable overall quality for the longest storage duration; however, berries stored at temperatures higher than 0°C had more aroma compounds and higher antioxidant capacity during the postharvest period (Ayala-Zavala et al., 2004). A significant reduction in total aroma volatile production was observed in mature green mango fruit (*M. indica* L. "Kensington Pride") stored at 0, 5, 10, or 15°C compared with fruit stored at 20°C (Nair et al., 2003). The degree of reduction in aroma volatile compounds depended on the severity of chilling injury caused by bad temperature management. Carrots (*Daucus carota* L.) were processed into shreds and stored for up to 4 months at −24°C (frozen storage), or the roots were stored for up to 4 months at 1°C (refrigerated storage) followed by processing into shreds. A considerable increase in aroma volatiles was observed during refrigerated storage, whereas the aroma volatiles were around the same level during frozen storage (Kjeldsen et al., 2003). Biolatto et al. (2005) reported that sensory characteristics, such as sweet, acid, and bitter taste and typical flavor intensity, were not affected by cold quarantine treatments of grapefruit (*Citrus paradise* Macf.). Therefore, this treatment and temperature management may have important commercial applications for grapefruit without adversely affecting its quality. The effect of storage temperature on the chemical composition and sensory quality of custard apple (*Annona squamosa* L.) fruits stored at 10, 15, 20, and 25°C was studied (Prasanna et al., 2000). The texture, taste, and flavor of ripe fruits held at 25 and 20°C were superior followed by fruits stored at 15°C. Cooling before ripening at 20°C led to the best flavor of peach fruit (*P. persica* L.) without excessive total losses. These results helped in the optimization of warming cycles during cold storage used to avoid chilling-injury development on peaches (Fernandez-Trujillo et al., 2000).

14.4.5 Physicochemical treatments

Inhibition of physiological and/or pathological deterioration of fresh harvested produce by a combination of any type of physical, chemical, or environment-friendly chemical methods is called "physicochemical" treatments.

During fruit ripening on the tree and after harvest, some essential processes involve the production of the anaerobic metabolites, acetaldehyde, and ethanol. These processes include the production of aroma volatiles and removal of fruit astringency. A requirement for anaerobic metabolites in normal ripening provided the initial indication that the application of such anaerobic metabolites might be beneficial for postharvest fruit quality. In the postharvest period, anaerobic metabolites may be applied to induce volatile production and to improve fruit aroma (Pesis, 2005). "Murcott" mandarins held in N_2 exhibited a higher and an earlier increase in the accumulation of the off-flavor volatiles than "Star Ruby" grapefruit (Shi et al., 2005). Finally, sensory evaluations indicated that the taste of mandarins markedly deteriorated following exposure to anaerobic conditions and was rated as unacceptable after 48 h in N_2, whereas the taste of grapefruit deteriorated only slightly and was rated acceptable even after 72 h of exposure to N_2 (Shi et al., 2005). Fallik et al. (2005a) found that anoxia-treated tomatoes that were held at 20°C for 12 days had organoleptic qualities similar to those of untreated fruit that were held at cold storage plus marketing simulation, and the trained panel preferred the N_2-treated fruit. Pressure infiltration of apples with $CaCl_2$ solutions transiently inhibited volatile levels by forming a temporary barrier to CO_2 and O_2 exchange between the fruit tissue and the surrounding atmosphere (Saftner et al., 1999). Hypobaric (low pressure) storage offers considerable potential as a method to prevent postharvest loss of horticultural and other perishable commodities, such as fruit, vegetables, and cut flowers. The effects of hypobaric storage on the biological characteristics of green asparagus were compared against refrigeration and room temperature storage. Hypobaric storage maintained sensory quality and delayed the postharvest senescence process of asparagus (Li et al., 2006). Ozone is a disinfectant that can be applied by direct contact or by indirect contact through washing the produce with recycled water containing ozone. Ozone is a powerful oxidant that acts on carbon residues dissolved in the washing water, as well as on the produce surface. A detrimental effect of ozone treatment (0.35 ppm at 2°C) on strawberry aroma was observed, with a 40% reduced emission of volatile esters in ozonated fruits (Perez et al., 1999). The effect of ozone on sensorial quality of whole and fresh-cut tomatoes stored up to 15 days at 5°C was examined. In whole and sliced tomatoes treated with a humidified flow of ozone-enriched air applied cyclically (4 ppm of O_3), a higher sugar (fructose and glucose) and organic acid (ascorbic and fumaric) content was found (Aguayo et al., 2006). The O_3-treated fruit retained a good appearance and overall quality in slices, but had a reduced aroma. Ozone did not cause any damage or off-flavor in slices or whole tomatoes. In a different experiment, tomatoes were vacuum infiltrated at the breaker stage with ethanol (EtOH) vapor and then held for a further period before ripening in air at 22°C. Aroma or flavors were not altered as determined by a trained taste panel, except in extreme conditions, where in some cases off-flavors increased (Ratanachinakorn et al., 1999). Strawberries treated with methyl jasmonate (MJ) in conjunction with ethanol (MJ-ETOH) increased volatile compounds during storage period compared to control or fruit treated with ethanol alone. The postharvest life was longer for those berries treated with MJ-ETOH and MJ than for those treated with ethanol or control fruit (Ayala-Zavala

et al., 2005). Persimmon fruits (*Diospyros kaki* L.) were exposed to alcohol vapor in order to remove astringency, and their chemical and physical characteristics were measured. The best period for consumption of the fruits based on sensory quality was placed between the 4th and 8th day after the treatment, while control fruit became edible several days later (Antoniolli et al., 2000).

An alternative method of slowing ripening is treatment with 1-MCP, which inhibits ethylene action (Sisler and Serek, 1997). Exposure to 1-MCP inhibits ethylene production, and thus ripening, of climacteric fruits (Blankenship and Dole, 2003). The rapidly ripening summer apple cultivar "Anna" was treated with 1-MCP at harvest. Fruit quality was measured instrumentally at different times during the 20°C ripening periods, and compared to the sensory ratings. 1-MCP prevented softening and acidity loss in the treated apples, and this was reflected in increased preference by the sensory panelists. The highest preference was for 1 μL/L 1-MCP-treated apples after 12 days at 20°C (Pre-Aymard et al., 2005). Conference pears (*Pyrus communis* L.) were treated with 1-MCP, and then stored in air (NA) and controlled atmosphere (CA). After storage, fruits were retreated with 1-MCP. The 1-MCP effects were perceivable on texture (juiciness) and flavor. Control fruit and 1-MCP at 25 nL/L fruit reached their best sensory quality after 14 weeks of storage, while 50 nL/L fruit reached the same sensory quality later, keeping a fresh flavor when the quality of control fruit declined and became watery or grainy. CA storage prolonged or enhanced the effects of 1-MCP; 1-MCP cannot substitute for CA but can reinforce the CA effects (Rizzolo et al., 2005).

Irradiation has multiple benefits in food preservation through several processes such as disinfestations, delaying maturation, sprout inhibition, decontamination, and sterilization. Sensory evaluation studies in different commodities indicate that irradiation treatment does not affect quality and flavor (Patil, 2004). On the other hand, papayas, rambutans, and Kau oranges were irradiated at 0.75 kGy to determine the effect of X-ray irradiation on objective and sensory quality attributes (Boylston et al., 2002). The effects of irradiation and storage on specific sensory attributes were dependent on the specific fruit. Aroma and flavor tended to be more intense in the irradiated fruit.

14.5 Processed products

The production of fresh-cut fruit is increasingly becoming an important task as consumers are more aware of the importance of health, eating habits, and have less time for food preparation. A fresh-cut fruit is a fruit that has been physically altered from its original state (trimmed, peeled, washed and/or cut), but remains in a fresh state. Unfortunately since fruits are living tissue, they undergo enzymatic browning, texture decay, microbial contamination, and undesirable volatile production, highly reducing their shelf life if they are in any way wounded.

Maintaining flavor, aroma, and postharvest quality after processing and throughout the distribution chain is a major challenge facing the fresh-cut fruit industry. Development of fresh-cut products requires consideration of cultivars that store well as both intact and fresh-cut fruit. The sensory quality of two apple cultivars, Granny Smith and Fuji that were used for fresh-cut apples, was compared with that of two new cultivars, Pink Lady and GoldRush. The acceptability of flavor, texture, and overall eating quality of GoldRush slices was as good as that for Pink Lady and Fuji, compared to the commercial cultivars, Granny Smith and Fuji

(Saftner et al., 2005). The major volatile compounds and their concentrations in fresh-cut cantaloupe melon (*C. melo* L var *reticulatus*) decreased considerably with storage of the cut fruit for 24 h at 4°C (Lamikanra et al., 2003). Slight imbalances in compound concentrations may alter the overall perception of desirable, typical "cantaloupe" aroma/flavor during fresh-cut storage. Upsetting the unique aroma balance through storage may negatively affect flavor and the consumer's perception of desirable attributes, even though total volatile levels might not decrease substantially until after 5–7 days in storage (Beaulieu, 2005, 2006). Changes in postcutting sensory attributes during fresh-cut storage at 4°C in cantaloupe harvested at four distinct maturities (1/4, 1/2, 3/4, and full slip) were subsequently investigated (Beaulieu et al., 2004). Correspondingly, fruity and sweet aromatic flavor were significantly less intense in the 1/4-slip cubes compared with 1/2- and 3/4-slip maturities. Therefore, fresh-cut cantaloupe cubes with desirable sensorial attributes can be prepared with fruit when harvested greater than or equal to 1/2 slip but not from 1/4-slip fruit. Sensory evaluations indicate that precut heat treatment of cantaloupe at 50°C for 60 min increased intensities of desirable attributes such as fruity melon and sweet aromatic flavors, and reduced undesirable flavors such as musty, sour, bitter, chemical, and fermented (Lamikanra et al., 2005a). Postcut application of UV improved shelf life of cut cantaloupe melon; cutting fruit under UV-C radiation further improves product quality (Lamikanra et al., 2005b). Sensory aroma evaluation indicated reduced rancidity, and instrumental texture measurements suggested improved firmness retention in fruit cut under UV-C radiation.

Modified atmosphere packaging or edible coatings can be used to help in the preservation of minimally processed fresh produce, providing a partial barrier to moisture, oxygen, and carbon dioxide, improving mechanical handling properties, carrying additives, avoiding volatiles loss, and even contributing to the production of aroma volatiles (Olivas and Barbosa-Canovas, 2005). Beneficial effects of super atmospheric O_2 in regard to the sensory quality of other vegetable products have been reported (Day, 2000). The effect of super atmospheric O_2 (80 kPa) and MAP on organoleptic quality of minimally processed baby spinach was studied (Allende et al., 2004). Adding super atmospheric O_2 to the packages alleviated tissue injury in addition to reducing microbial growth and was beneficial in maintaining quality of fresh-cut baby spinach. Similar results were reported by Allende et al. (2002) for mixed salads using oxygen greater than 50 kPa. Five different packaging treatments, including two passive modified atmosphere packaging (MAP), two active MAP, and a moderate vacuum packaging (MVP), were used for minimally processed (MP) bunched onions (Hong and Kim, 2004). Sensory attributes of the onions were affected by packaging type. MVP with a gas-permeable plastic film retained better quality, with reduced microbial decay and visual sensory aspects, as compared with the other packages. On the other hand, fresh-cut cilantro (*Coriandrum sativum* L.) leaves that were packed inside a low gas-permeable plastic film developed a strong off-odor, accompanied by a rapid loss of typical aroma and overall visual quality, with an unacceptable quality rating at the end of 2-week storage at 0°C (Luo et al., 2004). Significant differences in sensory quality and aroma composition were found among the six cultivars of vacuum-packed, knife-peeled potatoes (Thybo et al., 2006). Storage period also affected the sensory quality and the aroma composition. Some of the aroma compounds were correlated to potato flavor and rancidity, whereas off-flavor/off-taste seemed to be correlated to nonvolatile components. The study showed that the quality of prepeeled potatoes is very sensitive to raw material quality, and the time of year being processed.

14.6 Future aspects and conclusions

While it is relatively straightforward to assess the economic benefits associated with reducing storage costs, and eliminating disorders that develop during storage of products, it is more difficult to assess the benefits associated by improving overall quality. Sensory quality is usually defined as all those characteristics of a food that lead a consumer to be satisfied with the product (Harker et al., 2003). However, it was concluded that taste, aroma, and freshness were most frequently chosen as decisive attributes for selecting fresh produce by consumers (Peneau et al., 2006), although quality may also be used to describe subjective attributes such as crispness, juiciness, flavor, or attractiveness (Barritt, 2001). Yet, problem exists in most agricultural crops, and has resulted in often legitimate consumer complaints concerning the lack of sensory quality (mainly aroma) in agricultural produce.

Fresh produce sensory quality is the sum of the interaction between sugars, acids, and a set of volatile compounds synthesized from a diverse set of precursors, including amino acids, lipids, and carotenoids. Some of these volatiles impart desirable qualities, while others are negatively perceived. Based on the data above, preharvest and postharvest factors were found to determine the overall external and internal quality if the fresh harvested and fresh-cut product. Moreover, sensory quality is also based on the manner in which the product is typically consumed and the means by which of the aroma compounds with the aromatic character impact are generated (Beaudry, 2000). However, it is impossible to point out which practices contribute more to the overall quality (Forney et al., 2000). Cultivar and site are two factors that affect fruit sensory quality where a grower's opportunity to influence that quality may be limited. A single decision made before the orchard is even planted regarding site and cultivar may have a profound affect on fruit quality throughout the life of the planting. This could be solved, in part, by getting the best possible cultivar using a breeding program. Crop breeding is used to improve plant variety and productivity in intensive agriculture.

Traditionally, most of the effort in breeding plants has been directed toward the inclusion of desirable agronomical traits, such as high yields, ease of mechanization, perfect visual appeal, plant resistance to pests and pathogens, enhanced shelf life, and other commercially important characteristics. It is unfortunate that with the development of these excellent crop varieties, traits that affect the aroma and flavor of fruit and vegetables have often been lost. Carbonell-Barrachina et al. (2006) suggested a different tool for breeding better fresh produce. A system specifically designed for the nondestructive analysis of volatile organic compounds in fresh tomatoes, based on a dynamic headspace technique, was used to quantify the volatile aroma constituents of the fruits. This system reduced the high variability associated to sample selection in postharvest studies. Volatile compounds with a major contribution to aroma are quantitatively determined in traditional cultivars and one commercial F1 hybrid, thus allowing the use of volatile determination as a possible tool in fresh fruit and vegetable breeding programs. Moreover, metabolic engineering can provide assistance in conventional breeding programs (marker-assisted breeding), or by the implementation of genetic engineering. Although the specific major flavor and aroma compounds have been identified in many fruits, the genes and enzymes involved are not yet fully understood. Thus, to implement the novel biotechnological advances in restoring the "lost" aromas of fruits, it is imperative to identify the genes that affect flavor and aroma production, and to understand their regulation and limitations. As a first step to identify the

genes responsible for the synthesis of flavor-related chemicals, an attempt is made to identify loci that influence the chemical composition of ripe fruits. Linked molecular markers should be useful for breeding programs aimed at improving fruit flavor. In the longer term, the genes responsible for controlling the levels of these chemicals will be important tools for understanding the complex interactions that ultimately integrate to provide the unique flavor of fresh or fresh-cut product (Tieman et al., 2006).

Besides preharvest factors, postharvest practices play major roles in determining sensory quality. Those practices combine many factors that will not only affect the storability and shelf life of the fresh or fresh-cut produce, but will determine sensory, acceptability, and marketability. Exporters, importers, and scientists are always looking for a reliable objective tool or measurement that will predict the best sensory quality of a superior cultivar together with the appropriate postharvest practices. These could be achieved by measuring aroma profiles using instruments in combination with organoleptic measurement as suggested by Fallik et al. (2001) for melons and Berna et al. (2005) for tomatoes.

Organoleptic quality involves taste and aroma, but also the color and texture of the fruit. Organoleptic cultivar classification based on groups' segregation such as balanced, tart, sweet or aroma will help to match ethnic preferences and enhance current promotion and marketing programs for better cultivar in regard to sensory quality, as suggested by Crisosto et al. (2006) for peaches and nectarines. Hoberg et al. (2003) concluded that with the aid of the human sensory method developed to characterize the melon varieties, it was possible to distinguish the different genotypes.

Variety, growing conditions, ripening stage, and storage conditions may influence the content of flavor and aroma volatiles, but little is known about the genetic control and the genes responsible for their variation (Fellman et al., 2000). Fresh produce breeders need selection criteria both efficient and easy to assess for organoleptic quality breeding. Physical and chemical traits could be an alternative approach for routinely measuring some of the quality traits, but molecular markers will provide a much more efficient tool. These results will be used for marker-assisted selection in order to transfer pleasant flavor characteristic of the new cultivar line into elite lines with better shelf life and sensory quality fruits. As few clusters can be detected and some QTLs (quantitative trait loci) will be shown to have strong effects, genetic progress is expected. In addition to the genetic work, sensory analysis is a tool that allows, with objective techniques, to evaluate the organoleptic properties of food products and to determine consumer acceptance. Both genetic and sensory tools will allow companies to understand the strengths and weaknesses of products; assist in the development of new products; modify and improve existing products; identify differences between analogous products, improve quality, assess storage conditions, and determine product shelf life.

The task of maintaining and improving the sensory quality of fresh and fresh-cut products will probably be difficult. Pre- and postharvest treatments currently used to reduce or prevent pathological deterioration, or to maintain texture and color, can compromise sensory quality. The task of improving our understanding through scientific inquiry appears discouraging. The metabolic pathways responsible for the synthesis of aroma compounds are diverse and often highly integrated with other portions of primary and secondary metabolism. However, some of specific proteins required for the biosynthesis of specific aroma have been characterized and the genes controlling its synthesis recently identified (Beaudry, 2000). As the genetic and biochemical factors that alter or control synthesis of

aroma volatile are better understood, pre- and postharvest tools to maintain, or improve the sensory quality should be developed better. These will allow development of fresh produce with longer shelf life and better flavor and aroma.

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Chapter 15

Polyamines and Regulation of Ripening and Senescence

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15.1 Introduction

Polyamines (PAs) are small polycationic, biogenic amines that have profound effects on growth, development, and senescence in eukaryotic cells (Galston and Kaur-Sawhney, 1995; Cassol and Mattoo, 2003; Casero and Marton, 2007). Diamine putrescine (Put) is a major PA in plants and a precursor for triamine spermidine (Spd) and tetraamine spermine (Spm). PAs influence many biochemical and physiological processes such as cell division, cell elongation, flowering, fruit set and development, fruit ripening, and senescence (Evans and Malmberg, 1989; Galston and Kaur-Sawhney, 1990; Bouchereau et al., 1999). Many of these processes have direct implications on various aspects of postharvest biology of fruit and vegetable crops including quality, storage life, senescence, chilling and other stresses, and disease development (Valero et al., 2002a). Significant information about PA action has emerged from indirect studies employing pharmacological levels of different PAs and as well as their biosynthetic inhibitors. In recent years, reverse genetics has begun to provide direct evidence for involvement of PAs in determining fruit quality, ripening, and processing attributes (Tiburcio et al., 1997; Martin-Tanguy, 2001; Mehta et al., 2002; Mattoo et al., 2006; Srivastava et al., 2007). Molecular aspects of PA action have also begun to emerge, especially from nonplant systems (Igarashi and Kashiwagi, 2006; Wallace and Niiranen, 2007). PAs have been shown to bind to membranes, nucleic acids, and other macromolecules, and have been implicated in stabilizing chromatin conformation, regulating ion channels, scavenging free radicals, and regulating gene expression (Casero and Marton, 2007; Srivastava et al., 2007). Since maintenance of membrane stability and homeostasis is essential for many cellular, physiological, and biochemical processes, it is likely that PAs can have a pivotal role in extending postharvest shelf life of fruits and vegetables. The focus of this chapter is on recent progress made in understanding the roles of PAs in plants with special emphasis on postharvest biological processes.

15.2 Regulation of polyamine metabolism in plants

15.2.1 Polyamine biosynthesis

The cellular concentration of different PAs ranges from micromolar to millimolar levels in plants and is highly regulated during growth and development (Imai et al., 2004;

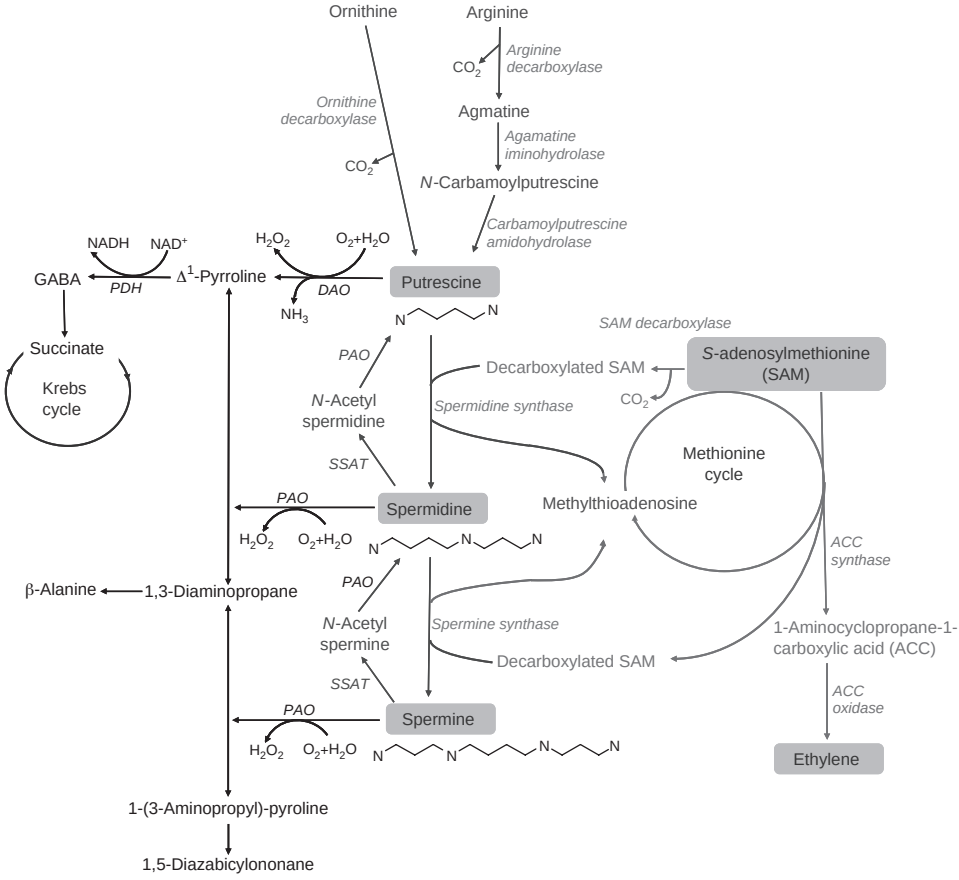

Fig. 15.1 Biosynthetic pathway of PAs: SAM is a common precursor of both PA and ethylene biosynthesis. Methionine cycle allows recycling of the carbon skeleton for biosynthesis of both PAs and ethylene, thereby preventing Met or SAM from becoming limiting under normal conditions. DAO, diamine oxidase; PAO, polyamine oxidase; PDH, pyrroline dehydrogenase; SSAT, spermidine/spermine acetyl transferase; GABA, γ-aminobutyric acid.

Gemperlova et al., 2006). As shown in Fig. 15.1, Put is synthesized in plants through either decarboxylation of ornithine by ornithine decarboxylase or decarboxylation of arginine to agmatine, which is subsequently converted to Put. The latter pathway is mainly present in bacteria and plants (Martin-Tanguy, 2001). In plants, agmatine is first converted into *N*-carbamoyl-Put by agamatine iminohydrolase, and subsequently *N*-carbamoyl-Put amidohydrolase converts *N*-carbamoyl-Put to Put (Mayer and Michael, 2003).

Ornithine-dependent Put production has been reported to be absent in *Arabidopsis* (Hanfrey et al., 2001), but present in other plants including tomato, tobacco, rice, and apples suggesting species-specific variation in PA production pathways. Put is converted to Spd by Spd synthase, and Spd is converted to Spm by Spm synthase, through sequential addition of aminopropyl residues derived from decarboxylated *S*-adenosylmethionine (dcSAM) (Mehta et al., 2002). dcSAM is synthesized from *S*-adenosylmethionine (SAM) by the catalytic activity of SAM decarboxylase. Levels of both Spd and Spm increase in transgenic tomatoes overexpressing SAM decarboxylase, demonstrating the availability of dcSAM as

a rate-limiting step for synthesis of Spd and Spm (Mehta et al., 2002). Cadaverine, a less widely distributed polyamine, is produced as a catabolic derivative of lysine through the activity of lysine decarboxylase (Bouchereau et al., 1999).

15.2.2 Polyamine and ethylene biosynthesis share a common precursor

Ethylene has tremendous impact on fruit ripening and has been termed as "ripening hormone" (Mattoo and White, 1991; Abeles et al., 1992; Fluhr and Mattoo, 1996). PA and ethylene biosynthetic pathways share a common intermediate, SAM. It has been suggested that competition for this substrate may tightly regulate internal levels of PAs and ethylene (Kushad and Dumbroff, 1991; Mattoo and White, 1991; Escribano and Merodio, 1994; Fluhr and Mattoo, 1996; Mehta et al., 1999; Pandey et al., 2000). Ethylene is a plant hormone that initiates fruit ripening in climacteric fruits and promotes senescence in leaves (see Mattoo and Suttle, 1991; Abeles et al., 1992; Mattoo and Handa, 2004). Ethylene has multiple influences on plant growth and development, in addition to enhancing ripening and senescence, thereby generating significant interest in understanding the interactions between ethylene and PAs in affecting various biochemical and physiological processes in fruits and vegetables. Ethylene is synthesized from SAM by sequential action of two enzymes: 1-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase. In vitro studies suggest that PAs inhibit ethylene biosynthesis in a variety of fruit and vegetative tissues, while ethylene suppresses the accumulation of PAs (Apelbaum et al., 1981; Li et al., 1992; Cassol and Mattoo, 2003). Inhibition of ethylene biosynthesis by PAs results in channeling of SAM into PA biosynthesis (Ben-Arie et al., 1982; Even-Chen et al., 1982; Roberts et al., 1984). These studies led to a hypothesis that a cross-talk exists between these two apparently antagonistic biosynthetic pathways (Mehta et al., 1997). However, increased ethylene production in tomato (Saftner and Baldi, 1990), cherimoya (Escribano and Merodio, 1994), and melon (Martinez-Madrid et al., 2002) is not always accompanied by a decline in Put. This suggests alternative interactions between ethylene and PA pathways in these systems. Watercore-affected apple fruit produced more ethylene and contained higher levels of Put, Spd, ACC, and 1-malonylamino-cyclopropane-l-carboxylic acid, a conjugate of ACC (Wang and Faust, 1992). Interestingly, accumulation of higher PAs, Spd, and Spm in transgenic tomato fruits expressing yeast SAM decarboxylase also resulted in several-fold higher ethylene production in these fruits as compared to the controls (Mehta et al., 2002). These results together suggest that PAs and ethylene pathways can be simultaneously active in fruits. Hence, more work is needed to show that production of SAM is actually a rate-limiting step in regulating levels of PA and ethylene in physiological processes (Matilla, 1996; Walden et al., 1997; Matilla, 2000; Mattoo et al., 2003).

15.2.3 Polyamine biosynthesis and methionine cycle

As mentioned above, methionine (Met) plays a central role in the production of PA and ethylene pathway. Methionine cycle facilitates recycling of Met from methylthioadenosine (MTA), a byproduct of PA and ethylene biosynthesis (Fig. 15.1) allowing for continued flux of Met into PA and ethylene (Wang et al., 1982; Yang and Hoffman, 1984; Miyazaki and Yang, 1987; Sauter et al., 2004; Katharina et al., 2007). MTA is depurinated by MTA nucleosidase to 5-methylthioribose (MTR) followed by phosphorylation of the C-1

hydroxyl group on the ribose moiety of MTR by MTR kinase yielding 5-methylthioribose-1-phosphate (MTR-P). MTR-P undergoes subsequent enzymatic isomerization, dehydration, and oxidative decarboxylation to 2-keto-4-methylthiobutyrate, which is the immediate precursor of methionine.

15.3 Polyamine catabolism

Amine oxidases such as diamine oxidase (DAO) and PA oxidase (PAO) are primarily responsible for PA catabolism (Fig. 15.1). DAO oxidizes Put to pyrroline, hydrogen peroxide, and ammonia. Pyrroline can be further catalyzed to form γ-aminobutyric acid (GABA) that is subsequently converted to succinate and incorporated into the Krebs cycle, thus recycling carbon and nitrogen from Put. PAO catalyzes production of pyrroline and 1,5-diabicyclononane, from Spd and Spm, respectively, and generates diaminopropane (DAP) and hydrogen peroxide. DAP subsequently produces β-alanine (Bouchereau et al., 1999; Tavladoraki et al., 2006). Association of amine oxidases with primary and secondary cell wall tissues during specific developmental processes has been reported (Rea et al., 2004). Hydrogen peroxide released through these catabolic reactions has been suggested to control processes such as lignification, suberization, and cell wall stiffening. In vertebrates and yeast, acetylated forms of Spm and Spd can be converted back to Put by the activity of Spd/Spm acetyl transferase (SSAT). Activity of SSAT has not yet been demonstrated in plant tissues. However, acetyl PAs are present in sugar-beet seedlings (Christ et al., 1989), *Helianthus tuberosus* chloroplasts (Del-Duca et al., 1995) and various organs of *Arabidopsis*, suggesting that PA interconversion may also occur in plants (Tassoni et al., 2000). Also, yeast and animal Spm oxidases can oxidize Spm back to Spd, suggesting SSAT-independent back conversion of PAs (Cona et al., 2006). An *Arabidopsis* PAO was shown to catalyze production of Spd and nor-Spd from Spm and nor-Spm, respectively (Tavladoraki et al., 2006).

15.3.1 Conjugated and bound polyamines

PAs can exist as free, bound, and conjugated forms in most plant systems. PAs are conjugated to hydroxycinnamic acids by an amide linkage using esters of CoA catalyzed by a class of enzymes called transferases. They occur both as basic and neutral forms. In the basic form, one amine group of PAs is associated with phenolic cinnamic acid, and in the neutral form each terminal amine group of an aliphatic amine is linked with cinnamic acid (Martin-Tanguy, 1997). Spd, homo-Spd, and Spm conjugate with fatty acids as well as cinnamoyl compounds (Martin-Tanguy, 2001). Conjugation of PAs, through binding of hydroxycinnamic acid, is thought to have important roles in long-distance translocation of PAs and floral induction (Martin-Tanguy, 1985, 1997). Owing to their cationic nature, PAs are bound with several proteins such as transglutaminases (discussed later in the chapter) and other macromolecules in tobacco, oats, and petunia (Apelbaum et al., 1988; Mizrahi et al., 1989).

15.4 Uncommon polyamines

Several uncommon PAs such as homo-Spd, aminopropyl-cadavarine, thermo-Spm, nor-Spd, nor-Spm, caldopentamine, homocaldopentamine, caldohexamine, and homocaldohexamine

occur in microorganisms and have been speculated to offer protection in mediating growth responses under extreme environmental conditions (Kuehn et al., 1990). Occurrence of such uncommon PAs has also been reported in an osmotic stress-tolerant strain of alfalfa where Spd synthase give rise to thermospermine in addition to Spd and Spm (Bagga et al., 1997). PAs also lead to the formation of several alkaloid compounds of pharmacological interest in many plants. Compounds such as tropane alkaloids and nicotine are derived from Put through the activity of Put *N*-methyl transferase (Hibi et al., 1994). Additionally, alkaloids can also be derived through conjugation products of other PAs (Smith et al., 1983). Piperidine and quinolizidine alkaloids are derived from cadaverine (Wink and Hartman, 1982).

15.5 Polyamine transport and localization

Long-distance transport of PAs has been reported in plants, but the presence of a PA transporter has not yet been demonstrated (Martin-Tanguy, 1985). However, radiolabeling of zucchini hypocotyls with [^{14}C]-Spd showed binding to 66-kDa and 44-kDa plasma membrane proteins suggesting the presence of putative Spd-binding polypeptides in plants (Tassoni et al., 1998). However, multiple PA transporter genes have been reported in *Escherichia coli* and yeast (Kashiwagi et al., 1990, 1997; Tomitori et al., 2001).

The ODC pathway for PA synthesis was predominantly localized to the cytoplasm in the algae, *Chlamydomonas reinhardtii* (Voigt et al., 2000). In higher plants, both nuclear and cytoplasmic localization of this enzyme has been reported (Slocum and Flores, 1991). In tobacco, ADC is mainly localized to the chloroplast of photosynthetic tissues such as leaves and stems and to the nucleus in nonphotosynthetic tissues like roots and flowers, suggesting its role in photosynthesis and cellular signaling processes such as protein phosphorylation. ADC does not possess transmembrane domains and hence, localization to thylakoid membranes may be due to a predicted putative amphiphilic α-helix-forming region with high hydrophobicity (Borrell et al., 1995). Spd synthase has been localized to the chloroplast (Cohen et al., 1981) and cytoplasm (Sindhu and Cohen, 1984). Additionally, the PA catabolic enzyme PAO was localized to the cell wall (Kaur-Sawhney et al., 1981; Slocum and Furey, 1991).

15.6 Polyamines, regulators of postharvest processes

PAs have been implicated in a multitude of growth and developmental processes such as embryogenesis, root formation, floral initiation and development, pollen tube growth, fruit development including ripening, senescence, biotic, and abiotic stress responses (Slocum and Flores, 1991; Cohen, 1998; Cassol and Mattoo, 2003; Kaur-Sawhney et al., 2003). Fruit ripening and senescence are two processes that are closely associated with the shelf life of fresh produce and produce destined for processing. In vitro and pharmacological experiments with PAs have led to the suggestion that PAs are important plant growth regulators with the ability to inhibit ripening and senescence in a number of plant tissues (Galston and Kaur-Sawhney, 1995; Cassol and Mattoo, 2003). PAs may also play a significant role by regulating biotic and abiotic stress responses that limit quality and postharvest shelf life of produce.

15.6.1 Role of polyamines in fruit set, growth, and development

Normally, higher levels of PAs are present in flowers but decrease upon fertilization (Pritsa and Voyiatzis, 2004). Flower opening in damson plum is coincident with increases in free PA levels in the sepals, petals, and sex organs (Dios et al., 2006). In ovaries, Spm level decreases following fertilization, with a concomitant increase in Put and Spd in the sepals. Spd is the most abundant PA during flowering, at S1 and S2 fruit growth phases, whereas Put levels are highest during S3 and S4 fruit growth phases (Dios et al., 2006). Both arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) activities decrease following tomato fruit set. There is also an indication that the ADC pathway may be involved in cell expansion and the ODC pathway active in cell division process during early fruit growth of tomato (Cohen et al., 1982; Alabadi et al., 1996; Alabadi and Carbonell, 1998). Application of Put, Spd, and Spm to wild-type unpollinated ovaries in tomato resulted in partial parthenocarpy (Fos et al., 2003). Higher PA levels in unpollinated *pat*-2 ovaries are correlated with the activation of the ODC pathway, which in turn is influenced by elevated GA levels found in these ovaries (Fos et al., 2003). Several other studies have also implicated PAs in the initial stages of fruit development, which include cell division and cell elongation (Table 15.1).

Table 15.1 shows patterns of changes in free PAs during growth of indicated climacteric and nonclimacteric fruits. The levels of Put, Spd, and Spm are generally high during early fruit growth but decline during later phases of fruit development (Valero et al., 2002a). Higher levels of PAs are observed during the period when growth is primarily accomplished by cell division. Levels of PAs during early fruit development in tomato, peach, and plum are higher, with Put being the most abundant PA. Application of ODC inhibitors impairs fruit growth indicating a role for PAs in regulating cell division and fruit development (Egea-Cortines and Mizrahi, 1991; Fos et al., 2003). The levels of PAs decline at the later stages of fruit growth and development (Saftner and Baldi, 1990; Morilla et al., 1996; Shiozaki et al., 2000; Mehta et al., 2002; Alburquerque et al., 2006; Liu et al., 2006a). In tomato, levels of Put and Spd declined between immature green and mature green stages (Saftner and Baldi, 1990). Over 100-fold higher Spd levels were present during cell division than at later stages of fruit growth in tomato (Egea-Cortines et al., 1993). However, there are some exceptions as increase in Put in muskmelon and in Put and Spd in long-keeping tomato were observed during fruit growth.

15.6.2 Polyamines and ripening

From a postharvest view point, ripening increases consumer acceptability by rendering fruit attractive and palatable through enhanced organoleptic characteristics such as flavor, texture, color, and aroma. Fruit ripening is a genetically programmed process and is accompanied by a major shift in gene expression leading to desirable metabolic processes (Giovannoni, 2004; Srivastava and Handa, 2005). Based on respiration patterns at the onset of ripening, fruits are classified into either climacteric or nonclimacteric fruits. Climacteric fruits exhibit increased respiration during ripening, whereas in nonclimacteric fruits respiration rate decreases as fruits ripen. Ethylene is necessary for ripening of climacteric fruits as inhibition of either ACC synthase or ACC oxidase by reverse genetics impairs progression of the ripening

Table 15.1 Changes in PA levels during fruit growth and ripening phases

	During initial growth phase			During continued fruit growth			During ripening phase			
Fruit	Put	Spd	Spm	Put	Spd	Spm	Put	Spd	Spm	References
Climacteric fruit										
Peach cv. Biscoe	Full bloom			7 weeks AFB			14 weeks AFB			Kushad (1998)
	357	535	218	130	110	100	110	110	100	
	↑	↑	↑	↓	↓	↓	↓	≅	≅	
Muskmelon cv.	20 DAA			35 DAA			52 DAA			Lester (2000)
Honey dew	0	250	NA	75	275	NA	250	150	NA	
	↓	↑		↑	≅		↑	↓		
Tomato cv. Indalo	0.01 g of FW			1 g of FW			100 g of FW			Morilla et al.
	1,800	600	500	130	200	65	75	60	10	(1996)
	↑	↑	↑	↓	↓	↓	↓	↓	↓	
Nonclimacteric fruit										
Strawberry cv.	0 DAP			10 DAP			25 DAP			Ponappa and
Allstar	155	200	125	130	60	50	10	40	5	Miller (1996)
	↑	↑	↑	↓	↓	↓	↓	↓	↓	
Pepper	7 DAFS			35 DAFS			100 DAFS			Serrano et al.
	2,700	610	310	1,050	300	200	>50	>50	>50	(1995)
	↑	↑	↑	↓	↓	↓	↓	↓	↓	
Eggplant cv.	9 DAPF			13 DAPF						Rodríguez et al.
Black nite	18	5	NA	3	3	NA	NA	NA	NA	(1999)
	↑	↑		↓	≅					
Tomato cv.	Immature green			Mature green			Ripe			Dibble et al.
Alcobaca	300	35	NA	100	40	NA	350	40	NA	(1988)
	↑	↓		↓	↑		↑	≅		

All PA values are nmol/g fresh weight.
↓ = decreases, ↑ = increases, ≅ unchanged, NA = not available.
Development stages are indicated as follows: AFB, after full bloom; DAA, days after anthesis; FW, fresh weight; DAP, days after pollination; DAFS, days after fruit set; DAPF, days after petal fall.

process (Oeller et al., 1991; Picton et al., 1993). Nonclimacteric fruits such as eggplant, citrus, grapes, and strawberry do not seem to need ethylene to complete the ripening process (Valero et al., 2002a). A nonclimacteric long-keeping tomato mutant showed an increase in Put during ripening, while in strawberry and pepper PA levels decreased during ripening. In climacteric fruits such as tomato (cv. Indalo) and peach, the levels of polyamines decreased during the course of fruit ripening; however, Put increased during ripening in muskmelon (Table 15.1). Transgenic tomato expressing yeast SAM decarboxylase under a ripening specific promoter showed increases in Spd and Spm at the cost of Put and exhibited extended *on planta* fruit life (Mehta et al., 2002). This shows a variation in developmental trends of PA levels across different fruit systems.

Fruits with slow and fast ripening and short and long shelf life have been used to understand the interplay between PAs and ethylene during postharvest storage. In tomato, total PAs declined during growth of slow (Liberty) and fast (Pik Red and Rutgers) ripening tomato varieties. While PA decline persisted in Rutgers and Pik Red, varieties with short storage

life, it increased during ripening in Liberty, a variety with prolonged shelf life (Saftner and Baldi, 1990). Liberty also contained three to six times more PAs than Rutgers and Pik Red and produced only 16 and 38% of ethylene produced by Pik Red and Rutgers, respectively. Application of 1-methylcyclopropene (1-MCP), an inhibitor of ethylene perception, delayed ripening in tomato fruits, and reduced free Put levels that increased when these fruits started to ripen, suggesting a role of PAs during fruit ripening (Tassoni et al., 2006b). Similar trends were observed in other slow- and fast-ripening tomato cultivars (Martinez-Madrid et al., 1996). Japanese pear cultivars with longer shelf life exhibit higher levels of PAs and show a negative correlation between PA levels and the rate of ethylene production (Mora et al., 2005). In nectarines, exogenous application of Put and Spd reduced ethylene production, delayed loss of firmness, retained titratable acidity, and prevented the increase in dry matter and soluble solids concentration (Torrigiani et al., 2004). Put treatment also downregulated expression of ACC oxidase and SAM decarboxylase at the transcriptional level suggesting that applied PAs affect fruit ripening by altering ethylene biosynthesis. On the other hand, transgenic tomato expressing yeast SAM decarboxylase showed increased biosynthesis of both PAs and ethylene, indicating that endogenous level of SAM is not rate limiting for either pathway during fruit ripening (Mehta et al., 2002). Based on the effects of 1-MCP on free Put levels and expression of its biosynthetic enzymes, Tassoni et al. (2006b) reached a similar conclusion.

Carotenoid metabolism in tomato that is tightly linked to ripening is associated with differentiation of chloroplasts into chromoplasts and is extensively regulated at the transcriptional level (Bramley, 2002; Cookson et al., 2003). Tomato fruit overexpressing the yeast SAMdc had increased conversion of Put into higher PAs with severalfold increase in Spd and Spm in ripening fruits. These fruits exhibited a two- to threefold increase in lycopene (a nutritionally important antioxidant), prolonged vine life, and enhanced fruit juice quality (Mehta et al., 2002). Although these studies suggest a role for PAs in influencing carotenoid metabolism, further studies are needed to probe a direct link between PAs and carotenoid metabolism.

15.7 Polyamines and postharvest shelf life

Ripe fruit of "*alcobaca*" landrace tomato variety exhibited prolonged keeping qualities and contained three times more Put than a control variety, "Rutgers" (Dibble et al., 1988). As both genotypes showed similar catabolism of Put and Spd, increased ADC activity has been suggested to be the cause of elevated levels of Put in *alcobaca* fruits (Rastogi and Davies, 1991). Transgenic tomato fruits that accumulate higher levels of Spd and Spm due to the expression of SAM decarboxylase showed prolonged vine life (Mehta et al., 2002). Vacuum infiltration of tomato fruit with Put, Spd, Spm, diaminopropane, γ-aminobutyric acid, and methionine increased their storage life (Law et al., 1991). Pomegranate fruits treated with Put or Spd, either by pressure infiltration or immersion, and subsequently stored at 2°C for 60 days had higher levels of ascorbic acid, total phenolic compounds, and total anthocyanins in arils than the untreated samples (Mirdehghan et al., 2007b).

Hot water dips and chilling induced decay in plum and decreased ethylene production while increasing PA levels during subsequent storage at 0°C (Abu-Kpawoh et al., 2002). Put infiltration of four different plum varieties delayed ripening and extended shelf life at

20°C. In addition, Put increased shelf life, enhanced fruit quality attributes such as soluble solids, titratable acid content, and fruit firmness and resulted in either a decrease or a delay in ethylene production (Perez-Vicente et al., 2002; Serrano et al., 2003). Loss of fruit firmness in peach during postharvest storage is associated with a decrease in the levels of PAs and an increase in ethylene biosynthesis (Liu et al., 2006b). Exogenous treatment of field-grown peaches with PAs and aminoethoxyvinylglycine (AVG), an inhibitor of ethylene biosynthesis, inhibited ethylene emission, delayed flesh softening, and retained titratable acidity (Bregoli et al., 2002; Torrigiani et al., 2004). Since these compounds affect ethylene production and expression of genes involved in ethylene biosynthesis, this hormone is likely to influence the mode of action by which these PAs affect shelf life (Torrigiani et al., 2004).

It is important to note that the method of treatment, the cultivar used, and the stage of ripening can influence the effect of PAs in altering postharvest qualities. Vacuum infiltration with Put or dipping in a solution of Put decreased ethylene production by kiwi fruit and delayed flesh firmness in contrast to pressure infiltration (Petkou et al., 2004). Comparison of Put and Spd treatments showed differential effects on the production of ethylene and fruit quality attributes between attached fruit and fruit explants in peaches and nectarines (Bregoli et al., 2006). Treatment of potted miniature roses with various PAs and methylglyoxal-guanylhydrazone, a PA-synthesis inhibitor, did not modify floral longevity (Serek and Andersen, 1994).

15.7.1 Polyamines and postharvest fruit firmness

Treatment with exogenous PAs has been shown to increase flesh firmness in several fruits including “Golden Delicious” and “Mclntosh” apples (Kramer et al., 1991), strawberry slices (Ponappa et al., 1993), plums (Perez-Vicente et al., 2002), peaches and nectarines (Bregoli et al., 2006). However, differential effects of Put, Spd, and Spm have been reported. For example, vacuum infiltration of Spd and Spm significantly increased firmness of strawberry slices, whereas Put was ineffective (Ponappa et al., 1993).

Some of the effects of PAs on fruit firmness could be due to modification of genes involved in ethylene biosynthesis, ethylene perception, alteration of cell wall–associated enzymes, and PA conjugation. Prestorage application of Put suppressed ethylene biosynthesis probably by lowering ACC oxidase (ACO) enzyme activity and delayed fruit softening in plum by modulating cell wall–related enzymes involved in fruit softening such as pectin esterase, exopolygalacturonase and endopolygalacturonase (Khan and Singh, 2007). Also, in vitro inhibition of pectin methylesterase and polygalacturonase by PAs has been reported (Leiting and Wicker, 1997; Martinez-Tellez et al., 2002). Calcium and heat-treated plums stored at lower temperatures also showed increased fruit firmness, which may be due to the effect of conjugated Put and Spd (Valero et al., 2002b). The role of PAs in delaying softening in peach and apple can be attributed to their effects on stabilization of cell walls and membranes (Bonghi et al., 1998; Liu et al., 2006a, b). Higher levels of cell wall–bound Put and Spd contributed to increased firmness and reduction in mechanical damage in plum (Perez-Vicente et al., 2002). PAs bind to pectins associated with the cell wall, thus influencing modifications of cell wall in addition to altering the binding of other ions such as calcium (Messiaen et al., 1997). Further cross talk between ions such as calcium and PAs may also influence the activity of pectin esterase (Leiting and Wicker, 1997).

15.8 Polyamines and senescence

PAs have been linked with cell death-related processes in many organisms and are considered as juvenility factors (Thomas and Thomas, 2001; Serafini-Fracassini et al., 2002). In many plant systems, leaf and fruit aging and senescence is correlated with a decrease in PA levels. Exogenous application of PAs often delays or prevents progression of senescence (Kaur-Sawhney et al., 1982; Sood and Nagar, 2003). Tomato fruits treated with UV (3.7×10^3 J/m^2) showed delayed ripening and senescence, which was attributed in part to the maintenance of a high level of Put (Maharaj et al., 1999). Treatment of hypodermal-mesocarp tissues of "Honey Dew" melon fruits with Spd or Spm reduced membrane peroxidation indicated by lower production of malondialdehyde, decreased lipoxygenase and phospholipase-D activities, and decreased perturbation of plasma membrane as indicated by higher H^+-ATPase activity (Lester, 2000). These data support a role for membrane lipid and PA interaction during senescence. A PA conjugate, *N*-4-hexanoyl-Spd, accumulates in senescing petals and ovaries of pea. Conjugation of Spd with hexanoic acid can reduce the positive charge and affect interactions with anionic groups present on membrane phospholipids and nucleic acids, leading to membrane destabilization and senescence (Seiler, 1987; Perezamador et al., 1996). PAs, mainly Spm, retarded the senescence of leaf discs of two diverse species of rose, whereas PA synthesis inhibitors such as difluoromethylarginine (DFMA) and methylglyoxal-bis-guanylhydrazone (MGBG) promoted senescence. This retardation of senescence by PAs may be due to the inhibition of enzymes such as peroxidase and cellulase (Sood and Nagar, 2003). Spd-inhibited fruitlet abscission in grapevine increased soluble sugar content but reduced the levels of amino acids. These studies indicate a direct or indirect link of PAs with regulation of abscission via sugars and/or amino acids (Aziz, 2003).

Cut carnation flowers, treated with 10 mM Spd, exhibited a delay in senescence. The treated petals accumulated free and PCA-soluble Spd, which correlated with stabilization and reduced degradation of DNA (Tassoni et al., 2006a). Spermine was also shown to delay senescence of cut carnation flowers through reduction of ethylene production (Lee et al., 1997) as previously shown for other systems (Apelbaum et al., 1981). In the ovary of senescent *Hibiscus* flowers, the levels of PA-conjugates bound to small molecules decreased (Seo et al., 2007). Senescence in nodules is mainly associated with loss of ability to fix nitrogen, due to decreased nitrogenase activity, and with lower leghaemoglobin (LB) levels. Analysis of developing root nodules in five genotypes of *Vigna* showed a peak in nitrogenase activity, LB content, and levels of free Put during flowering. This strong correlation between nitrogenase activity, LB, and free Put suggests a role for Put-mediated sequestration of N_2 through nitrogenase in nodules (Lahiri et al., 2004).

15.9 Polyamines and abiotic stress response

PAs have been implicated in alleviating stress-induced injury to various fruit crops. Pomegranate fruit treated with Put and Spd under pressure infiltration or immersion exhibited reduced chilling injury and delayed deterioration of fruit quality (Mirdehghan et al., 2007a). Treatment of fruit with GA (gibberellin) and Put increased fruit firmness and reduced susceptibility to mechanical stress in peaches with a concomitant reduction in respiration and ethylene production (Martínez-Romero et al., 2000). These effects were attributed to an increase in Spd levels rather than an indirect effect of respiration or ethylene. Increased

firmness and delayed color development in GA-vacuum infiltrated or heat-treated (45°C) lemons were associated with increases in free Put and Spd during storage at 15°C (Valero et al., 1998). GA-treated lemons also showed a decrease in ABA (abscisic acid) suggesting a possible interaction between PA and ABA in delaying senescence. Thus, there appears to be a cross talk between PAs and various hormones in modulating these physiological responses (Srivastava and Handa, 2005). Evidence for direct roles of PAs has begun to emerge from transgenic model plants such as tomato, tobacco, *Arabidopsis*, and rice. In such instances, overexpression of PA biosynthetic genes was shown to impart tolerance of the transgenic plant to various abiotic stresses such as salt, drought, cold, dehydration, and K^+ deficiency (Capell et al., 1998, 2004; Roy and Wu, 2001; Kumria and Rajam, 2002; Perez-Amador et al., 2002; Urano et al., 2003; Waie and Rajam, 2003; Armengaud et al., 2004; Hummel et al., 2004; Alcazar et al., 2006; Wi et al., 2006).

PAs may ameliorate oxidative stress in plants as they are known to scavenge free radicals such as superoxide (O_2^-) and hydroxyl radicals (OH^-) formed enzymatically or chemically (Drolet et al., 1986). Hydrogen peroxide generated due to PA catabolism is required for lignification and cross-linking of extensins in response to stress and wounding (Cona et al., 2006). A PA-interacting protein showed 60% identity with catalase suggesting an interaction of PAs with the ROS pathway (Votyakova et al., 1999). Pretreatment with Spd prevented increase in hydrogen peroxide (H_2O_2) and NADPH-dependent superoxide in microsomes and protected a chilling-sensitive cucumber from chilling injury (Shen et al., 2000). *Arabidopsis* plants overexpressing *Cucurbita ficifoli* Spd synthase gene showed tolerance to paraquat, an oxidative stress inducer (Kasukabe et al., 2004). Also, PAs protected sunflower leaf discs against oxidative stress induced by metals (Groppa et al., 2001).

Exogenous application of Spd to oat plants subjected to osmotic stress showed stabilization of native structure of thylakoid proteins D1 and D2, cytochrome b_{559}, and Rubisco, indicating protection of the photosynthetic machinery. This effect could be mediated through diaminopropane formed during PA catabolism (Besford et al., 1993). PAs regulated the voltage-dependent inward K^+ channel, KAT1 in the plasma membrane of the guard cells, causing a decrease in stomatal aperture, which suggests a role for PAs in stress response through stomatal regulation (Liu et al., 2000). In addition, synthesis of uncommon PAs and interaction with various hormones may also play a role in stress adaptation. However, various signal transduction mechanisms involved in plant responses to abiotic stresses are still not fully understood. Hence, effects of PAs in stress responses should be interpreted with caution, and further information regarding the mechanisms involved is required.

15.10 Polyamines and biotic stress response

An increase of cell wall–bound PAs concomitant with a decrease in free PAs has been reported in diseased organs of *Vitis vinifera* infected with *Eutypa lata* (grapevine dieback). Levels of conjugated PAs decreased in diseased and increased in healthy organs in response to eutypiosis, which suggested a role for PAs in responses to *E. lata* infection (Rifai et al., 2004). Treatment of apples with three PA biosynthesis inhibitors, α-DFMO, DFMA, and α-methylornithine (MeOrn), individually or in combination with $CaCl_2$, reduced growth of *Botrytis cinerea* and *Penicillium expansum* and reduced soft rot symptoms associated with these pathogens (Saftner et al., 1997).

15.11 Molecular basis of polyamine effects

15.11.1 Cellular effects of polyamines

PAs affect many cellular processes including cell proliferation, cell division and differentiation, apoptosis, homeostasis, gene expression, protein, and DNA synthesis (Tabor and Tabor, 1984; Slocum and Flores, 1991; Cohen, 1998; Igarashi and Kashiwagi, 2000). However, in spite of these varied effects of PAs, little is understood about the mode of action at the cellular level. All these physiological roles suggested for PAs in fruit growth, ripening, shelf life and stress tolerance may be partly due to their cationic nature that facilitates PAs binding to proteins and membranes. PAs also bind to nucleic acids and may thereby regulate gene expression and modulate homeostasis as reflected by changes in global gene expression (Srivastava et al., 2007) and modulation of metabolite (Mattoo et al., 2006) and protein profiles (Mattoo et al., 2007). Understanding the cellular roles of PAs in mediating these physiological parameters is important to comprehend the nature of PA action. However, studies of cellular roles of PAs in plants are limited, and current knowledge of their mode of action is derived largely from non-plant systems.

15.11.2 Interaction of polyamines with DNA, RNA, and proteins

Spd and Spm can bind to DNA and modulate their stability and conformation, and influence chromatin remodeling (Raspaud et al., 1999; Childs et al., 2003; Keniry, 2003). Triplex DNA exhibited highest aggregation in the presence of PAs, but higher concentration of PAs resolubilized these aggregates with single-stranded DNA-PA complex being the easiest to solubilize (Childs et al., 2003). Molecular dynamics simulations and UV absorption studies indicate that PAs preferred stabilization of A-DNA over B-DNA conformation and also induced B-Z transition (Bryson and Greenall, 2000). Uranyl photoprobing was used to show that PAs preferentially bind to bent adenine tracks of double-stranded DNA (Lindemose et al., 2005). PAs have been implicated in increasing core nucleosome stability and also in facilitating in vitro condensation of nucleosomal fibers (Makarov et al., 1987; Morgan, 1987). Nuclear aggregates of PAs have been suggested to play a crucial role in protecting genomic DNA and its conformation (Agostino et al., 2005). About 90% of Spd and 50% of the Put exist as PA–RNA complexes in *E. coli* (Igarashi and Kashiwagi, 2006). PAs control phosphorylation of nucleolar proteins in pea (Datta et al., 1987). Covalent binding of PAs possibly through the activity of transglutaminases (TGases) may aid in storage and transport of these compounds. A role for PAs in buffering cellular pH has also been proposed (Galston and Kaur-Sawhney, 1995).

15.11.3 Polyamine interactions with membranes

PAs may influence fruit ripening, senescence, improvement of fruit shelf life including firmness through their effects on stabilization of membranes and prevention of protein, nucleic acid, and chlorophyll degradation, processes that are associated with senescence (Ben-Arie et al., 1982; Galston and Kaur-Sawhney, 1987; Evans and Malmberg, 1989; Brüne et al., 1991; Pandey et al., 2000). Anti-senescence role of PAs in membrane stabilization is attributed to their ability to bind and interact with negatively charged phospholipid

components or other anionic sites on membranes (Hong and Lee, 1996). PAs bind, and stabilize membranes and proton-secreting systems of oat protoplasts (Galston and Kaur-Sawhney, 1995). In senescing barley and oat leaves, external application of PAs stabilized thylakoid membranes and prevented chlorophyll loss (Popovic et al., 1989; Besford et al., 1993). Exogenous application of Spd to barley leaf discs inhibited RNase activity, loss of chlorophyll, and protein degradation from thylakoid membranes, thus stabilizing them during senescence (Legocka and Zajchert, 1999). Reduction in loss of chlorophyll and reduced levels of malondialdehyde in oat leaves supplied with Spd, Spm, diaminopropane, and guazatine (an inhibitor of PAs oxidase) suggested an anti-senescence role of PAs through inhibition of lipid peroxidation (Borrell et al., 1997).

PAs conjugated to Ca^{2+}-dependent transamidating enzymes, TGases, play an important role in preventing senescence by stabilizing proteins. TGases catalyze the formation of cross-linkages between glutaminyl- and lysyl-residues or between glutaminyl-residues and PAs, thus forming mono- or bis-PA derivatives. Transglutaminase activity measured in terms of formation of mono- and bis-derivatives of Put and bis-derivatives of Spd from corolla of undetached flowers of *Nicotiana tabacum* indicated an increase in mono-PAs and a decrease in bis-derivatives during early senescence. Also, application of Spm to excised flowers delayed senescence and cell death while increasing mono-Put (Serafini-Fracassini et al., 2002).

15.11.4 Polyamines and gene expression

PAs can alter gene expression both at the transcriptional as well as the translational level. PA depletion may stabilize mRNA due to lack of Spd for post-translational modification of eIF5A, an important factor involved in mRNA turnover (Mehta et al., 1991; Veress et al., 2000). In *E. coli*, PAs influence proteins synthesis at the level of translation through the PA modulon (Yoshida et al., 2004; Igarashi and Kashiwagi, 2006). Expression of certain genes including transcription factors constituting the PA modulon is altered by PAs at the level of translation. Microarray analysis of a PA-requiring *E. coli* mutant showed that a large number of genes involved in cellular processes such as central intermediary metabolism, energy metabolism, iron, and zinc transport processes were upregulated by PAs. The PA modulon influenced 58 of the 309 upregulated genes. Genes downregulated by PAs were mainly associated with amino acid metabolism, biosynthesis of cofactors, prosthetic groups, and carriers (Yoshida et al., 2004; Igarashi and Kashiwagi, 2006).

Genes associated with different stress signaling pathways such as transcription factors involved in salt, cold, and dehydration responses were downregulated in plants overexpressing ADC. Additionally, *AtGA20ox1*, *AtGA3ox1*, and *AtGA3ox3* genes involved in GA biosynthesis and pathogen response were upregulated (Alcazar et al., 2005). Under chilling stress, transgenic *Arabidopsis thaliana* overexpressing SpdSyn showed upregulation of many genes including transcription factors involved in chilling tolerance. Other genes upregulated in these transgenic plants encode protein kinases, calmodulin-related proteins, cytochrome P450, and peroxidases. However, most of the downregulated genes did not appear to be related to stress tolerance (Kasukabe et al., 2004).

NMR spectroscopy–based metabolite profiling revealed distinct metabolite trends in high polyamine transgenic tomato fruits, overexpressing SAM decarboxylase compared to control fruits. Gln, Asn, choline, citrate, fumarate, malate, and an unidentified compound

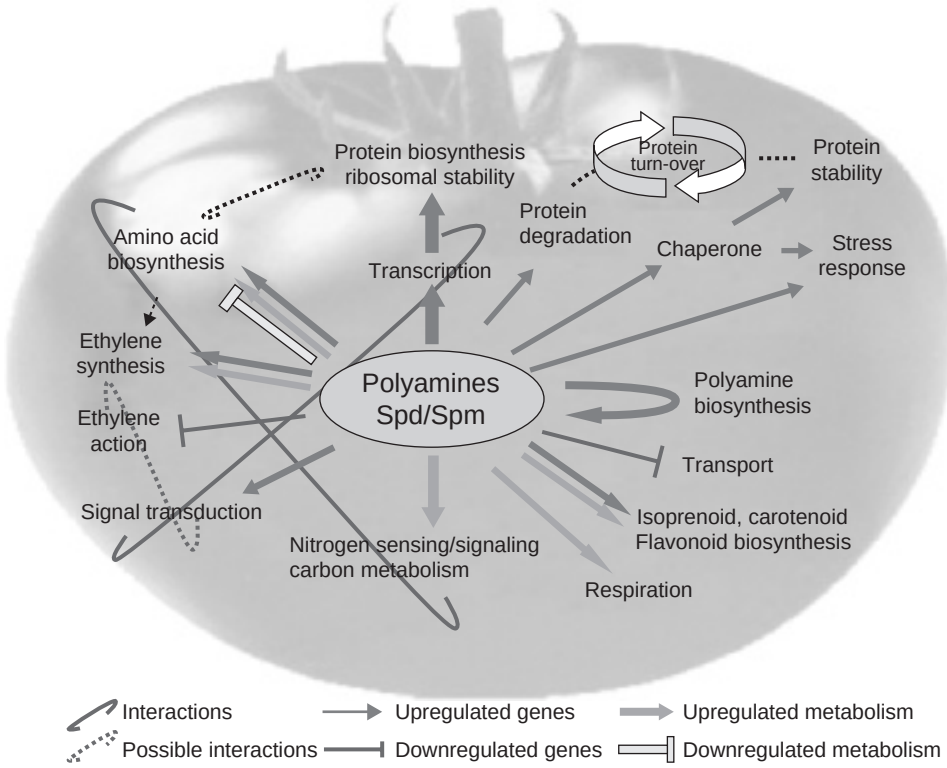

Fig. 15.2 Diagrammatic representation of pathways/process regulated by Spd/Spm in tomato fruit as revealed by macroarray analysis metabolic profiling in tomato. (Redrawn from Srivastava et al., 2007.)

A accumulated, while the levels of Val, Asp, sucrose, and glucose decreased in the red transgenic fruit compared to the control red fruit. These results indicated that pathways involved in nitrogen sensing/signaling and carbon metabolism are preferentially activated in transgenics with high Spd/Spm but low Put (Mattoo et al., 2006). Transcriptome analyses of these transgenics indicated upregulation of over 20% genes representing many different anabolic pathways, supporting an anti-senescence role of PAs (Srivastava et al., 2007). Most of the differentially expressed genes represented functional categories involved in transcription, translation, signal transduction, chaperone activity, stress responses, amino acid biosynthesis, ethylene biosynthesis and action, PA biosynthesis, and isoprenoid and flavonoid biosynthesis. Figure 15.2 illustrates a composite of transcriptome analysis and altered metabolite profiles. Taken together, these results show that PAs significantly enhance anabolic pathways and thus may act as anabolic growth regulator(s) in plants (Mattoo et al., 2007; Srivastava et al., 2007).

15.12 Concluding remarks

Biological revolution led by merging genetics with biotechnology and pyramiding of genes is a promising development that will create designer crops with better quality (phytonutrients), longer shelf life, and traits conferring resistance to postharvest pathogens and abiotic

stresses. Although a number of chemicals, including polyamines, have been reported to improve various desirable traits, more knowledge base is needed in order to apply this technology to create plants precisely tailored to prevent fruit or produce loss normally incurred during harvesting, handling, transportation, storage, and marketing. The focus of this chapter has been to explore the role of PAs, particularly in influencing processes related to postharvest shelf life of produce. Although much information has been generated, clear understanding of the mechanisms of action of PAs is still in its infancy. Comprehensive information regarding PA uptake, long-distance transport, and subcellular localization is not complete. Is the role of PAs direct or manifested indirectly through their physical nature and/or via other hormones? What are the signal transduction pathways downstream of PA recognition, transport, and binding to functional components? The emerging technologies should help substantially in addressing the role PAs play in postharvest biology which can, in turn, be applied to increase both quality and shelf life of fresh produce. These include genetic and biochemical approaches involving identification and characterization of mutants altered in their response to PAs; using biosynthetic inhibitors and understanding the mechanism of inhibition; recombinant DNA-based transgenic approaches coupled with global gene expression analyses; metabolomics; and unraveling cross talks between PAs and other plant growth hormones and regulators. These studies should ultimately lead to rational design and strategic manipulation of biotechnological tools for enhancing valuable postharvest traits in fruit and vegetable crops. Already steps are in place to lead us into the inner core of PAs function, and depending on the nature of the focus, we may take one step at a time or bypass several by strategizing logarithmic jumps. The dye is cast!

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Chapter 16

Postharvest Enhancement of Phenolic Phytochemicals in Apples for Preservation and Health Benefits

Kalidas Shetty, Ishan Adyanthaya, Young-In Kwon, Emmanouil Apostolidis, Byungjin Min, and Paul Dawson

16.1 Introduction

Phenolic phytochemicals are secondary metabolites synthesized by plants, which constitute an important part of the diet in both humans and animals (Mann, 1978; Bravo, 1998; Crozier et al., 2000). In plants, these phenolics exhibit protective functions against environmental and biological stress such as high-energy radiation exposure, bacterial infection, or fungal attacks (Briskin, 2000). In addition, phenolics are also important for cell structure, signaling, and pigmentation. Due to diversity of functions, phenolic phytochemicals are expressed in diverse cell and tissue types (Briskin, 2000; Shetty, 1997; Vattem et al., 2005). Since phenolics have been linked to many protective functions in plants, it is likely they play similar protective roles in fruits; making them a key factor in postharvest preservation of fruits.

In addition, growing evidence suggests that the intake of phenolics via fruits and vegetables is linked to reduced risk of chronic diseases like diabetes, cardiovascular disease, and cancer (Hertog and Feskens, 1993; Hertog et al., 1995; Knekt et al., 1997, 2002; Boyer and Liu, 2004). A study with many popular fruits found apples to contain the second highest total phenolics content after cranberries, but the highest soluble phenolics (Sun et al., 2002). Apples also had the second highest free radical-scavenging-linked antioxidant activity among the fruits investigated (Sun et al., 2002). Apples are a popular fruit all over the world and have the potential to contribute as a significant source of phenolic antioxidants. In the United States, 22% of phenolic intake via fruit comes from apples (Vinson et al., 2001). In Finland the main source of dietary phenolics is onions and apples, and in the Netherlands, tea, onions, and apples are the biggest sources of phenolics (Hertog and Feskens, 1993; Knekt et al., 1997). Since the emerging oxidation-linked diseases can benefit from high intake of fruits and vegetables, high-phenolic antioxidant-containing apples have promise for enhancing human health through cellular protective functions.

There are more than 180 million people worldwide who have diabetes, and 90% of these suffer from non-insulin-dependant type 2 diabetes (World Health Organization, 2006). The World Health Organization (WHO) estimates that the number of people with diabetes will

double by 2030 (World Health Organization, 2006). The WHO states that type 2 diabetes can be prevented by physical activity, healthy eating, and prevention of obesity (World Health Organization, 2006). In addition, WHO figures show that about 80% of people with type 2 diabetes are from low- and middle-income countries (World Health Organization, 2006). Therefore, there is a need for low cost and easily available methods for management of diabetes in order to improve the quality of living of most diabetes patients.

A number of previous studies have found phenolics from many common foods like capsicum, cinnamon, and fenugreek to have the relevant phytochemical profile of, α-glucosidase inhibition and low α-amylase inhibition coupled with free radical-scavenging-linked antioxidant activity, for potential diabetes management (McCue et al., 2005; Kwon et al., 2006, 2007). This offers the potential for good postprandial blood glucose management via α-glucosidase inhibition without the common side effects associated with high α-amylase inhibition (McCue et al., 2005; Kwon et al., 2006, 2007). In addition, these same foods have free radical-scavenging-linked antioxidant activity, which can help maintain the redox balance in susceptible cells (McCue et al., 2005; Kwon et al., 2006, 2007). Since apple is a common fruit with no known side effects, any α-glucosidase-inhibiting effects, if found, are promising for type 2 diabetes management.

Based on the above background, we have explored the potential duel benefit of apple phenolics for better postharvest preservation and health benefits. Specifically, phenolic-linked changes were investigated during postharvest storage of apples. The objective was to determine the changes in phenolic content over the storage period and its relevance to postharvest preservation and concurrently determine any anti-diabetes-linked health benefits that could be attributed to the phenolic content. The health-relevant parameters investigated were in vitro antioxidant activity and inhibition of α-glucosidase and α-amylase relevant for glycemic index modulation. In addition, understanding of how inducible phenolics and related antioxidant activity (both free radical and enzyme linked) are coupled to the pentose phosphate pathway with positive consequences for postharvest preservation of apples was investigated. This was done by evaluating antioxidant enzyme activity, proline content, phenolic content, and free radical-scavenging antioxidant activity, and activity of key enzymes over a 3-month postharvest storage period. The enzymes evaluated were glucose-6-phosphate dehydrogenase (G6PDH), succinate dehydrogenase (SDH), proline dehydrogenase (PDH), guaiacol peroxidase (GPX), superoxide dismutase (SOD), and catalase (CAT).

16.2 Synthesis, functions, and health benefits of phenolics

Phenolic phytochemicals are synthesized by a common biosynthetic pathway that incorporates precursors from both the shikimate and/or the acetate-malonate pathways (Mann, 1978; Strack, 1997). The first step in the synthesis of phenolic phytochemicals is the commitment of glucose to the pentose phosphate pathway (PPP), converting glucose-6-phosphate irreversibly to ribulose-5-phosphate. This first committed step in the conversion to ribulose-5-phosphate is carried out by glucose-6-phosphate dehydrogenase (G6PDH). The conversion to ribulose-5-phosphate also produces reducing equivalents (NADPH) for cellular anabolic reactions (Fig. 16.1). PPP also generates erythrose-4-phosphate that along with phosphoenolpyruvate, from glycolysis, is channeled to the shikimate pathway to produce phenylalanine, which is directed through the phenylpropanoid pathway to produce

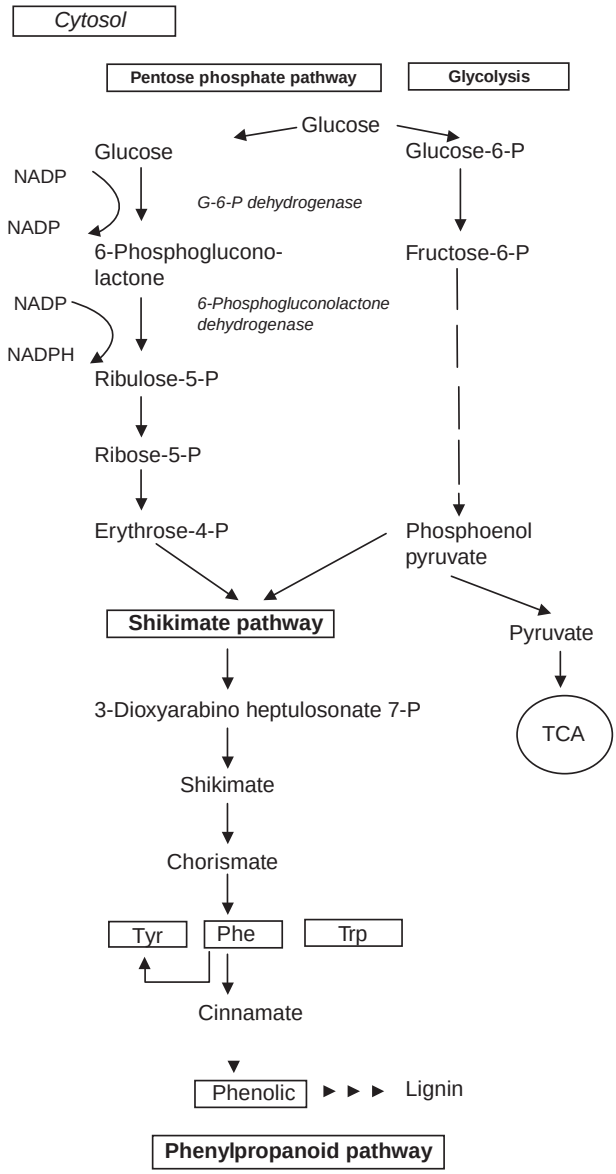

Fig. 16.1 Biosynthesis of phenolic phytochemicals.

phenolic phytochemicals (Mann, 1978; Chugh and Sawhney, 1999; Shetty et al., 2003; Shetty and Wahlqvist, 2004; Vattem et al., 2005; Fig. 16.2).

These phenolic phytochemicals have been shown to have a wide array of functions in plants. In plants biotic and abiotic stress has been shown to stimulate secondary metabolite synthesis that results in the production of phenolics (Dixon et al., 1994; Dixon and Paiva, 1995). Ozone exposure has been shown to increase transcript levels of enzymes involved in the phenolic synthesis and lignin pathways (Brooker and Miller, 1998). Studies have linked

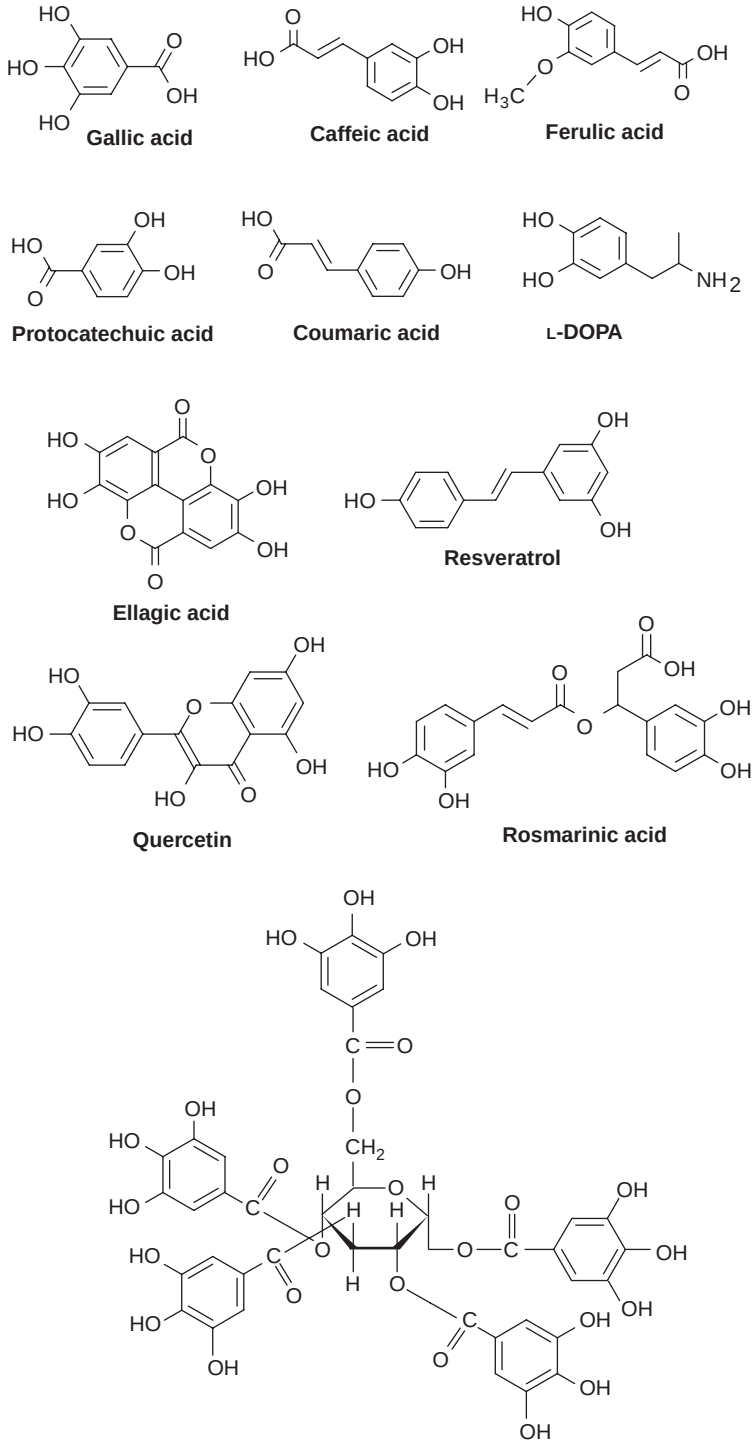

Fig. 16.2 Common simple phenol, biphenyls, flavanoids, and tannins in plants.

increase in thermotolerance during hyperthermia to accumulation of heat shock proteins and phenolic metabolites (Zimmerman and Cohill, 1991). Many phenolics are induced in response to infection, wounding, nutritional stress, cold stress, and UV irradiation (Rhodes and Wooltorton, 1978; Beggs et al., 1987; Hahlbrock and Scheel, 1989; Christie et al., 1994; Dixon et al., 1994; Lois and Buchanan, 1994; Dixon and Paiva, 1995). Phenolics can function as effective antioxidants by scavenging singlet oxygen and free radicals via their ability to donate hydrogen from hydroxyl groups positioned around the aromatic ring (Hertog et al., 1992; Foti et al., 1994; Hertog et al., 1995; Rice-Evans et al., 1995; Jorgensen et al., 1999). A recent study of antioxidant activity in apples by Lee et al. (2003) found that flavonoids like quercetin, epicatechin, and procyanidin B_2 rather than vitamin C contributed significantly to total antioxidant activity (Lee et al., 2003).

In light of the vast array of cellular protective functions phenolics have in plants, it is not surprising therefore that phenolics have diverse medicinal properties for human health applications. For example, curcumin from *Curcuma longa* and *Curcuma mannga* and rosmarinic acid from *Rosmarinus officinali* are used as antioxidants and anti-inflammatory compounds (Peake et al., 1991; Huang et al., 1992; Jitoe et al., 1992; Masuda and Jitoe, 1994; Osawa et al., 1995; Lim et al., 2001). Also, lithospermic acid from *Lithospermum* sp. is used as antigonadotropic agent, proanthocyanidins from cranberry can be used to treat urinary tract infections, and anethole from *Pimpinella anisum* is used as an antifungal agent (Winterhoff et al., 1988; Himejima and Kubo, 1993; Howell et al., 1998; Howell and Foxman, 2002).

Phenolic phytochemicals have also been found to have potential in the management of oxidative stress-linked chronic diseases like diabetes, cancer, and cardiovascular disease (Shetty, 1997, 1999, 2001; Shetty and Labbe, 1998; Shetty and Wahlqvist, 2004; Fig. 16.3). In a study involving Empire apples found phenolics from the apples to have potential cancer chemopreventive activity due to their combined antioxidant and anti-tumor-promoting activities (Kang et al., 2004). Similarly, an epidemiological study by Knekt et al. (1997) found that intake of dietary flavanoids showed an inverse association with lung

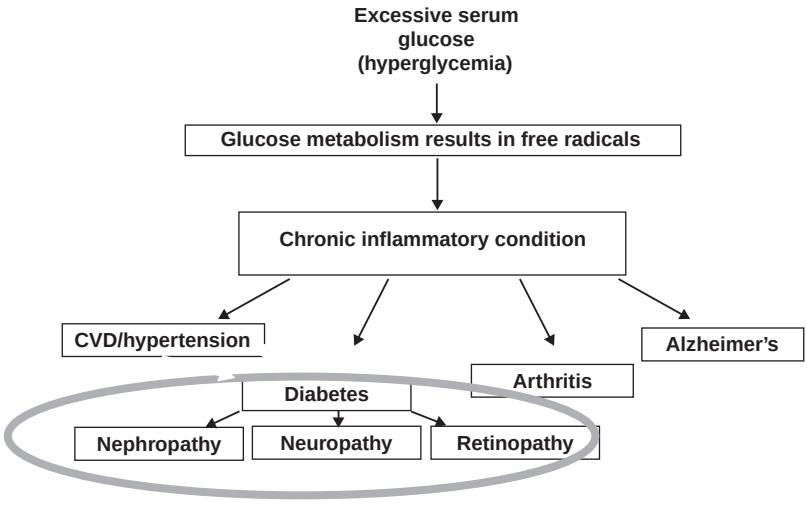

Fig. 16.3 Oxidative stress-related hyperglycemia and complications.

cancer incidence (Knekt et al., 1997). Another epidemiological study that examined the relationship between fruit and vegetable intake and the incidence of different cancers found that for most cancers the increase in risk for people with low fruit intake in the study was about twice as high as people with high intake (Block et al., 1992). In vitro antiproliferation studies with HepG2, human liver cancer cells, have shown phenolics from many common fruits like cranberry, lemon, apples, and strawberries to inhibit proliferation (Sun et al., 2002).

An epidemiological study by Knekt et al. (2002) of more than 10,000 Finnish men and women showed intake of foods containing flavanoids was associated with lower risk of type 2 diabetes. The study indicated the lower risk was associated with high intake of quercetin. The same study also states that the strongest association between high flavonoid intakes with lower risk for type 2 diabetes was seen when the source of flavonoids was from apples and berries (Knekt et al., 2002). Therefore, a diet containing apples has the potential to reduce risk of type 2 diabetes. Since apple is a common fruit with no known side effects, any α-glucosidase-inhibiting effects, if found, are promising for type 2 diabetes management.

16.3 Diabetes

Hyperglycemia has been linked to the onset of insulin-independent type 2 diabetes (Fonseca, 2003). A good strategy for management of type 2 diabetes is inhibition of enzymes that hydrolyze dietary polysaccharides in the gut, which can significantly reduce the rise in blood sugar levels after a meal by reducing the absorption of monosaccharides by the enterocytes of the small intestine (Puls et al., 1977; Ratner, 2001). Enzymes that hydrolyze dietary polysaccharides and modulate gut absorption are pancreatic α-amylase and α-glucosidases (Harris and Zimmer, 1992; Bischoff, 1994). However, a common side effect of these enzyme inhibitory drugs like acarbose is the excessive inhibition of pancreatic α-amylase, which can result in abdominal distention, flatulence, and diarrhea (Puls and Keup, 1975; Bischoff et al., 1985). These side effects are caused by abnormal fermentation of unhydrolyzed polysaccharides by gut bacteria (Puls and Keup, 1975; Horii et al., 1987). Therefore, in order to reduce these side effects but still manage hyperglycemia a high α-glucosidase inhibition and low α-amylase inhibition is beneficial.

Acute complications in patients suffering from type 2 diabetes are generally hyperglycemia-induced metabolic problems and infection. Long-term effects of hyperglycemia are microvascular complications like nephropathy, diabetic neuropathy, sexual dysfunction, and retinopathy and the macrovascular complication of hypertension (Nishikawa et al., 2000; Fig. 16.3). Recent studies have shown that hyperglycemia triggers generation of free radicals in mesangial cells in the renal glomerulus, neuron cells in peripheral nerves, and capillary endothelial cells in the retina (Brownlee, 2005). The generation of free radicals in all these cell types causes oxidative stress, which can be the cause of microvascular complications generally linked with hyperglycemia (Brownlee, 2005). Most cells are capable of reducing glucose transport inside the cell in hyperglycemic conditions so as to maintain a constant internal glucose level; however, the cells usually damaged by hyperglycemia were found to be inefficient in keeping their internal glucose levels constant (Kaiser et al., 1993; Helig et al., 1995). Therefore, it is not only important to control postprandial hyperglycemia but also keep in check any cellular redox imbalances to prevent diabetic complications.

In addition, another study has shown quercetin, which is a phenolic abundant in apples, to be an aldose reductase (alditol: NADP + oxidoreductase) inhibitor (Costantino et al., 1999). Aldose reductase is the first enzyme of the polyol pathway. Glucose metabolism through the polyol pathway has been linked to long-term diabetic complications like cataract, nephropathy, neuropathy, and retinopathy (Costantino et al., 1999).

16.4 Role of free radical-scavenging and enzyme antioxidant activity in postharvest preservation of fruits

Reactive oxygen species (ROS) like superoxide radicals ($O_2^{\cdot -}$), hydroxyl radicals ($\cdot OH$), and hydrogen peroxide (H_2O_2) are the products of oxidative dysfunctional biochemical reactions within cells. These ROS when left unchecked cause oxidative damage, resulting in lipid peroxidation, protein denaturation, and mutagenesis. An increase in ROS is linked to different types of stress such as drought, heat stress, metal toxicity, radiation exposure, pathogens, and salinity (Bolwell and Wojitaszek, 1997; Jimenez et al., 1998; Karpinski, et al., 1999; Dat et al., 2000; Hernandez et al., 2001; Quartacci et al., 2001; Del Rio et al., 2002; Fig. 16.4). Further ROS is also involved in natural and induced senescence and cell death in plants (Droillard et al., 1987; Thompson et al., 1991; Philosoph-Hadas et al., 1994; Bartoli et al., 1996). For example, studies have indicated increase in hydroperoxides during pepper, banana, pear, and tomato ripening during which senescence is induced (Frenkel, 1978; Thompson et al., 1987; Rogiers et al., 1998).

Plants counter harmful effects of ROS with antioxidants metabolites and enzymes. Antioxidants metabolites include water-soluble compounds like ascorbate, glutathione, and flavonoids and lipid-soluble compounds like carotenoids and tocopherols. Enzymes linked to antioxidant response include superoxide dismutase (SOD), catalase (CAT), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), ascorbate peroxidase (ASPX), and glutathione reductase (GR). SOD converts $O_2^{\cdot -}$ to H_2O_2, which is then reduced to H_2O by CAT or ASPX-depending cellular localization (Foyer et al., 1994; Hodges et al., 1996; Hodges and Forney, 2000; Fig. 16.5). Both ascorbate and glutathione can also interact directly with and scavenge ROS (Foyer et al., 1994). Oxidized ascorbate is reduced by DHAR, MDHAR, and glutathione, and oxidized glutathione is reduced by GR. Reduction of both oxidized glutathione and ascorbate by their respective enzymes is NADPH dependant (Shetty and Wahlqvist, 2004). Studies have shown many phenolic compounds especially flavonoids, for example, quercetin, rutin, and catechin have free radical-scavenging antioxidant activity (Kang et al., 2004; Zhang et al., 2006). In addition, studies in fava beans and peas have shown that even exogenous phenolics can stimulate antioxidant enzyme activity (Duval and Shetty, 2000; Vattem et al., 2005).

Therefore, since ROS is involved in plant development, including fruit ripening and senescence, antioxidant response coupling phenolic synthesis and antioxidant enzyme response may be recruited to counter ROS and senescence (Pastori and Del-Rio, 1997; Jimenez et al., 1998, 2002, 2003; Hodges and Forney, 2000). Previous studies with muskmelon fruits and sunflower seeds indicated that delayed senescence in specific tissue types correlated to high antioxidant enzyme response (Bailly et al., 1996; Lacan and Baccou, 1998). In order to couple cellular antioxidants like ascorbate, glutathione and phenolic phytochemicals with antioxidant enzymes for effective antioxidant response cellular reducing equivalents such as $FADH_2$ and NADPH are required. Studies have found that

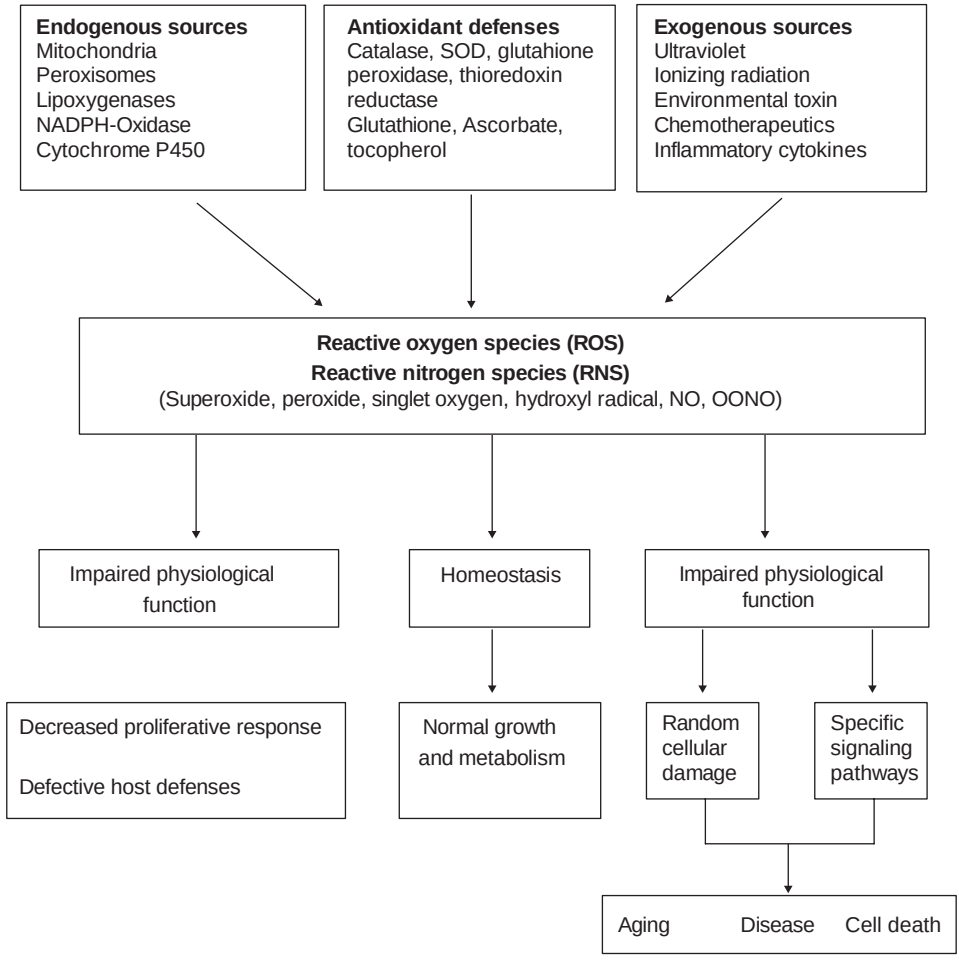

Fig. 16.4 Reactive oxygen species and cellular homeostasis.

reduced reductant levels increased the rate of senescence in a number of herbaceous species (Philosoph-Hadas et al., 1994; Meir et al., 1995).

16.5 Proline-linked pentose phosphate pathway for effective antioxidant activity and phenolic synthesis enhancing postharvest preservation

Another commonly seen stress response in plants is an increase in the synthesis of proline. Studies have shown proline biosynthesis to be stimulated in cases of water stress and salinity stress (Stewart and Larher, 1980; Thompson, 1980; Rhodes, 1987; Taylor, 1996; Hare and Cress, 1997). Proline synthesis and accumulation is also seen during senescence, freezing tolerance, water stress, salt stress, and dehydration in plant cells (Rhodes et al., 1986; LaRosa et al., 1991; Reddy and Veeranjaneyulu, 1991; Kiyosue et al., 1996; Sudhakar et al., 1993; Xin and Browse, 1998). It has been suggested that proline protects membranes in times of stress like those mentioned earlier (Paleg et al., 1981; Santoro et al., 1992).

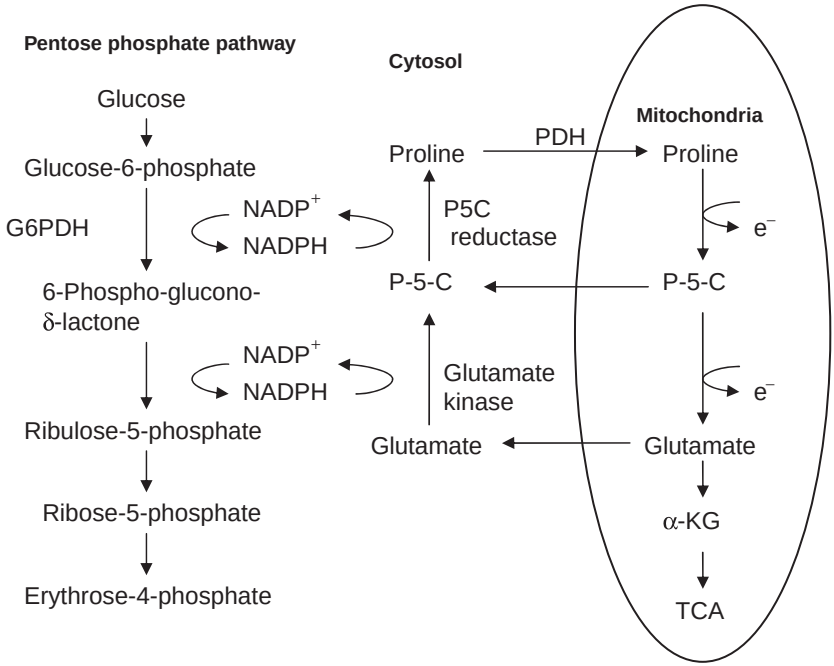

Fig. 16.5 Proline synthesis coupled to the pentose phosphate pathway. G6PDH, glucose-6-phosphate dehydrogenase; PDH, proline dehydrogenase; P-5-C, Δ^1-pyrroline-5-carboxylate; α-KG, α-ketoglutarate; TCA, tricarboxylic acid cycle.

An alternative model for coupling proline synthesis with the PPP has been proposed where proline biosynthesis in response to stress can manage energy and reductant needs of anabolic pathways (Shetty and Wahlqvist, 2004). This active metabolic role of proline could have implications for plant senescence where proline can act as an antioxidant or stimulate phenolic-linked antioxidant response (Smirnoff and Cumbes, 1989; Reddy and Veeranjaneyulu, 1991; Shetty and Wahlqvist, 2004). Proline is synthesized via the reduction of glutamate to Δ^1-pyrroline-5-carboxylate (P5C), which is further reduced to proline, with both reactions using NADPH as a reductant (Hagedorn and Phang, 1983; Phang, 1985; Shetty and Wahlqvist, 2004; Fig. 16.5). Since the reduction of P5C in the cytosol requires NADPH, an increase in the proline synthesis would result in a reduction in the NADPH/$NADP^+$ ratio, which has been shown to activate G6PDH (Lendzian, 1980; Copeland and Turner, 1987). G6PDH catalyzes the first rate-limiting step in the PPP; therefore, it is possible that during the postharvest storage period, different stress factors induce proline synthesis, which in turn stimulates the PPP (Shetty, 2004; Shetty and Wahlqvist, 2004).

This stimulation of the PPP would result in more essential reducing equivalents in the form of NADPH for efficient antioxidant enzyme response. The PPP stimulation would also make more sugar phosphate precursors, which along with the NADPH produced can support pathways for the synthesis of antioxidants, phenolic phytochemicals, and other protective compounds (Shetty, 2004; Shetty and Wahlqvist, 2004). The proline synthesized can function as an alternative reductant (in place of NADH) in mitochondrial oxidative phosphorylation to generate ATP (Phang, 1985; Shetty, 2004; Shetty and Wahlqvist, 2004) where proline dehydrogenase (PDH) catalyzes the first reaction of proline oxidation to

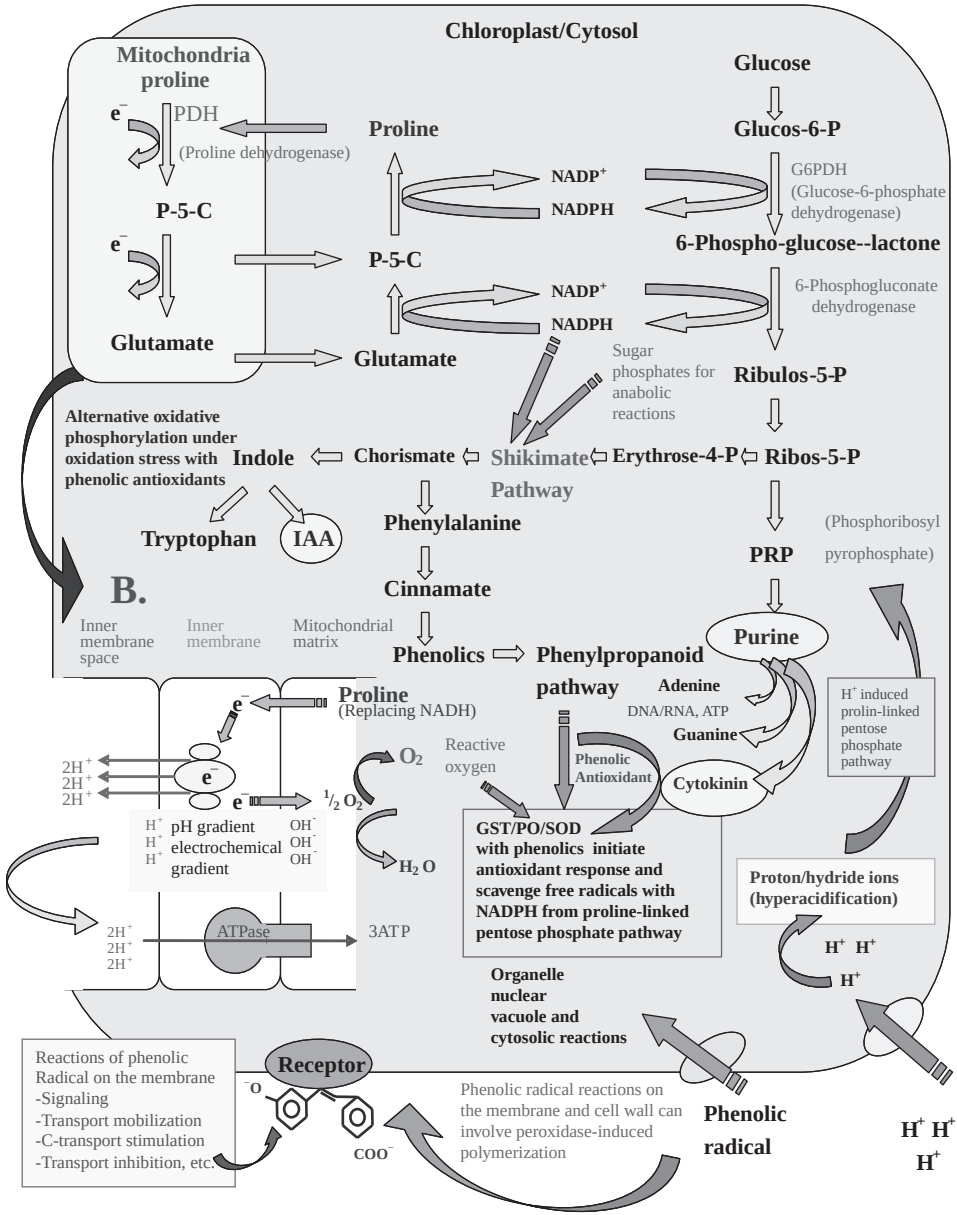

Fig. 16.6 Proline-linked pentose phosphate pathways for phenolic synthesis and efficient antioxidant response (Shetty and Wahlqvist, 2004).

P5C and linking the electron transport chain (ETC) in the mitochondria with oxygen as the terminal electron acceptor. P5C is then hydrolyzed nonenzymatically and oxidized to glutamate by the NAD-dependent P5C dehydrogenase. Following oxidative deamination by glutamate dehydrogenase, it flows into the TCA cycle through α-ketoglutarate to generate NADH for oxidative phosphorylation or recycled back into the cytosol (Hare and Cress, 1997; Shetty and Wahlqvist, 2004; Krishnan and Becker, 2006; Fig. 16.6).

16.6 Apple postharvest preservation is linked to phenolic phytochemicals and superoxide dismutase activity

The biochemical factors affecting postharvest preservation in apples indicated that well-preserved varieties of apples had increased SOD activity initially, and the activity declined during later storage as apples deteriorated (Adyanthaya et al., 2008a). The SOD link to better preservation correlated with higher phenolic content and free radical-scavenging-linked antioxidant activity. Well-preserved varieties were able to maintain a more stable pentose phosphate pathway (PPP) (measured by the activity of glucose-6-phosphate dehydrogenase, G6PDH) throughout the storage period. Proline content increased in all varieties with an increase in proline dehydrogenase (PDH) activity in the initial period indicating proline catabolism, supporting potential ATP synthesis. During later storage, SDH activity increased, while PDH activity declined indicating a shift to tricarboxylic acid cycle and likely NADH generation for ATP synthesis. This shift coupled with the declining SOD activity coincides with rapid deterioration. The GPX activity generally declined in late stages indicating postharvest deterioration.

16.7 Health benefits of apple phenolics from postharvest stages for potential diabetes management using in vitro models

Increasing number of studies has shown that regular intake of fruits and vegetables have clear links to reduced risk of chronic diseases like diabetes and cardiovascular disease. The beneficial effects in many cases have been attributed to the phenolic and antioxidant content of the fruits and vegetables. Apples are a major source of fiber and contain good dietary phenolics with antioxidant function. Previous epidemiological studies have indicated that intake of apples reduces the risk of developing type 2 diabetes. Our studies indicate that this reduced risk is potentially due to modulation of postprandial glucose increase by phenolics present in apples via inhibition of α-glucosidase (Adyanthaya et al., 2008b). Phenolic content was evaluated during 3 months of postharvest storage of four varieties of apples, and results indicated positive linkage to enhanced postharvest preservation and α-glucosidase inhibition. These in vitro results along with existing epidemiological studies provide strong biochemical rationale for further animal or human clinical studies.

16.8 Antioxidant and bioactive films to enhance food quality and phytochemicals production during postharvest stages

Consumers have many food choices, and their demand for safer and higher quality "natural" foods has increased. The entire food production process has a part in providing a more delicious, safe, and healthy food than in the past. Packaging plays an important role in maintaining quality and improving storage properties such as color, flavor, odor, microbial growth, and texture. In general, most foods are packaged to preserve quality. Films are important for safety since they come in direct contact with foods and the concept of packaging as only protection is gone. Now, packaging films play an active role in food preservation because it is possible to add physical, chemical, biochemical, and microbial modulators to films. Active packaging has been defined as "a type of packaging that changes packaging

conditions to extend shelf life, improve safety or maintain sensory properties of food" (Quintavalla and Vicini, 2002), and includes MAP (modified atmosphere packaging), antioxidant packaging, and other functional components. There is new interest in antioxidant films and in finding new potential antioxidant components. If more effective antioxidants can be successfully applied in films, another useful barrier can be created against oxidation, decomposition, and deterioration of foods. We propose the development of a new generation of active films and coatings. Current active packaging slows deterioration changes in food. The next generation of coatings will modify the biochemistry of raw foods to increase the concentration of positive health factors such as phenolic antioxidants.

16.9 Application of antioxidants films and commercialization

Due to the complexity of food, many factors influence the type of film and coating material to be used, including intrinsic food properties (pH, water activity, and composition) and extrinsic factors (temperature and relative humidity in processing and storage conditions) (Min et al., 2005). These factors may interact during processing or storage to give unexpected results. Due to the unpredictability of these interactions, commercialization of potential antioxidants in package materials and film forming is difficult. Packaging films incorporated with antioxidants can extend the shelf life of various foods including fruit and vegetables. For example, antioxidants can prevent or delay lipid peroxidation by binding iron ions and by stabilizing heme compounds, thereby stabilizing color, flavor, and sensory properties. For example, low-density polyethylene films with 0.1% tert-butyl-hydroxyanisole added resulted in higher "a" (redness) values in fresh beef compared to beef in control, tert-butyl-hydroxyanisole, rosemary extract, and α-tocopherol films by days 8 and 9 (Moore et al., 2003; Min et al., 2005).

16.10 Incorporation methods and migration of antioxidants

According to Quintavalla and Vicini (Quintavalla and Vicini, 2002), there are two common methods to incorporate components during film formation. The component can be placed into the film by addition to the extruder when the film is produced. The disadvantage of this method is poor cost-effectiveness since active components not exposed to the surface of the film are generally rendered inactive. An alternative to extrusion is to apply the active component in a controlled manner where the material is needed and not lost; for example, it can be incorporated into the food-contact layer of a multilayer packaging material. Also, active films can be classified in two types by migration methods: (1) those that contain an active agent that migrates to the surface of the food and (2) those that are effective without migration (Suppakul et al., 2003). While much of the research has been with antimicrobials, the antioxidants can be treated in the same manner. Active substances (antimicrobial or antioxidant components) can be added by different methods such as spray/coating before and after packaging, incorporation of active substances, and chemical immobilization after which substance can migrate to foods at different rates (Fig. 16.7; Min et al., 2005). Chemical immobilization can be performed by a nonfood agent. For successful application of antioxidant films with foods, various factors are to be considered such as water activity, pH, temperature, chemical interaction with film matrix, physical properties of packaging material, cost, and FDA approval.

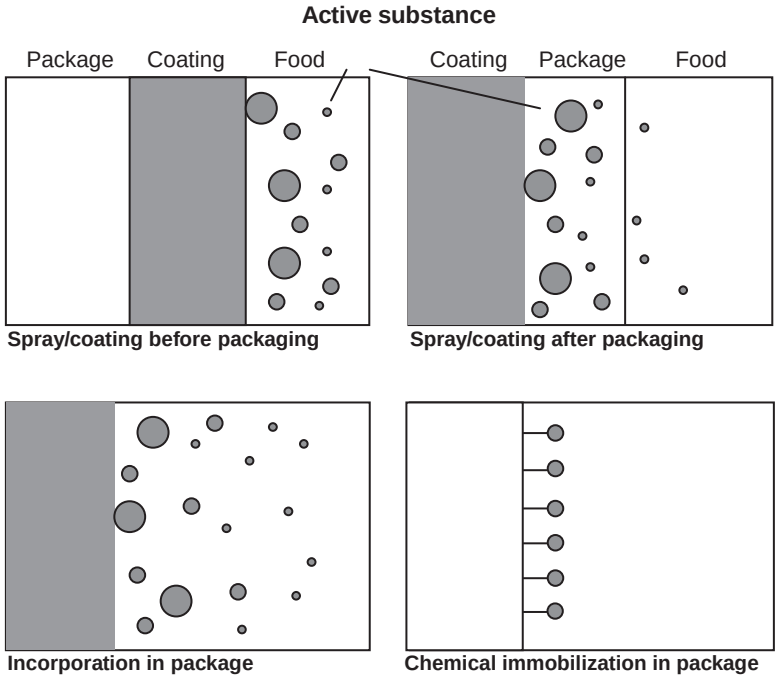

Fig. 16.7 Migration of active substance (Han, 2000).

16.11 Implications and future plan

To apply or correctly use antioxidant and bioactive packaging films against lipid oxidation and quality deterioration of foods, intrinsic and extrinsic factors of applicable foods affect to packaging materials must be measured. Because foods have different chemical and biological properties such as pH, water activity, carbon source, nitrogen source, partial pressure of oxygen, and temperature (Han, 2000), it is necessary to investigate a film's various material properties (heat resistance, moisture resistance, elongation, thickness, gas permeability), mass transportation activity, migration rate, and diffusivity. Interactions between packaging films and antioxidants and elicitors and whether the interaction affects original properties of foods will also be considered. After verifying interaction in vitro and in vivo without film malfunction, the film can be recommended for commercialization. A precise determination of standard activity as concentration of antioxidants or bioactivity in foods is needed to ensure high-quality-increased phenolic phytochemicals and safe foods in future markets. Research on new antioxidant and bioactive films can deliver safer and healthier foods to the table in the future.

Although there is abundant research on the effects and mechanism of discovered antioxidants in food systems, there is need to develop and apply new antioxidant components to improve food quality. Phytochemicals can be one of new developing components for film additives. Because phytochemicals have various functions such as antioxidation, anticancer, and bactericidal, we can place phytochemicals extracted from plant, natural fruit, and vegetables into packaging films to improve food safety or quality. Natural phenolic phytochemicals can be used in films not only for their intrinsic functional properties for

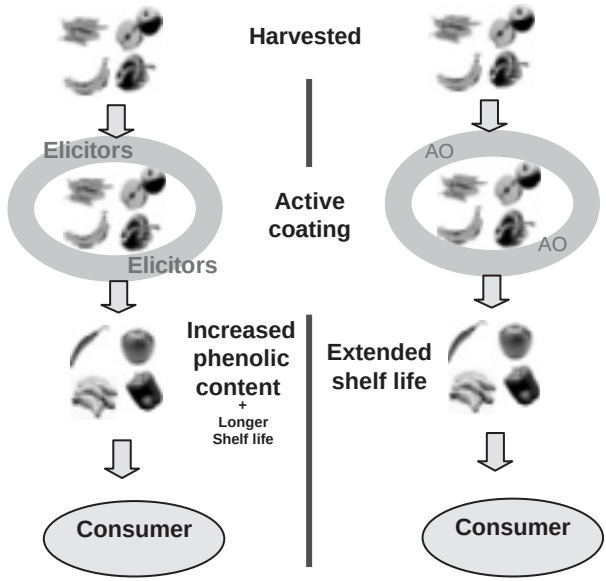

Fig. 16.8 Model of antioxidant films and coatings.

preservation (antioxidants and antimicrobials) and health benefits, but could also be used as exogenous elicitors of endogenous phenolics of fruits and vegetables that are targeted for preservation. Using this approach, the health-promoting functional phytochemicals of the preserved fruits and vegetables can be enhanced. There is evidence that exogenous phenolics can elicit endogenous phenolics in plant sprout systems (McCue et al., 2000; Randhir and Shetty, 2003; Shetty and Wahlqvist, 2004) and in clonal shoot cultures in response the polyaromatic hydrocarbons (Zheng et al., 1998, 2001; Zheng and Shetty, 2000). Further, empirical evidence implies that the endogenous natural phenolic biosynthesis in response to exogenous phenolics (synthetic or natural) could be regulated through an alternative proline-linked pentose phosphate pathway (Shetty and McCue, 2003; Shetty and Wahlqvist, 2004; Fig. 16.6). Therefore, it is our goal to use dietary phenolic phytochemicals from food-grade plants as components of film additives to (1) act as preservatives and enhance health property and (2) use a more innovative strategy to use same phenolic phytochemicals as exogenous elicitors to enhance endogenous health-relevant phenolics of the preserved fruits and vegetables through the proposed proline-linked pentose phosphate pathway (Shetty and McCue, 2003; Shetty, 2004; Shetty and Wahlqvist, 2004; Fig. 16.8).

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Chapter 17

Rhizosphere Microorganisms and Their Effect on Fruit Quality

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17.1 Introduction

This chapter summarizes some key aspects related to horticultural product quality when plants form symbiotic associations with beneficial rhizosphere microorganisms such as arbuscular mycorrhizal fungi (AMF) or plant growth–promoting rhizobacteria (PGPR). The development of those highly compatible associations brings a variety of benefits to nutritional status, health, growth, and development in plants. Several changes in host plant biochemistry and physiology occurring during these symbioses have been documented. Thus, fruit, vegetables, and grains may be modified not only in terms of biomass produced but also in some of their quality attributes.

The term "quality" implies the absence of defects or the degree of excellence of a product (Abbott, 1999), and it includes both sensory attributes that are readily perceived by the human senses and hidden attributes such as safety and nutrition (Shewfelt, 1999). The last objective of the production of fresh fruits and vegetables is to satisfy consumers, and consumer satisfaction is definitely related to quality; thus, good quality of horticultural products remains a prerequisite during production.

In the past few years, the changes in consumer attitudes and expectations associated with cultural practices and concerns about the side effects of agrochemicals have led to new perspectives on agricultural management. Research in this area has been oriented to developing technological alternatives that increase crop yield and improve product quality and lesser dependence on agrochemical inputs.

It is well known that the use of excessive nitrogen fertilizers is linked to nitrate contamination of ground water and emissions of ammonia and nitrogen oxides into the atmosphere (Bouwman, 1990; Houghton et al., 1990; Duxbury, 1994), while phosphate runoff in surface waters has led to eutrophication of ponds and rivers (Sharpley et al., 2003). Enhanced soil fertility and higher biodiversity found in organic plots may render agrosystems less dependent on external inputs (Mäder et al., 2002). On the other hand, while comparing organic and conventional production technologies, organic products have shown a significant increase in vitamin C, iron, magnesium, and phosphorus content and a decrease in nitrate content (Worthington, 2001). Additionally, the market tendencies indicated that the production of organic products represented US$23 billion in 2002 (Willer and Yussefi, 2004). Therefore, the development of organic agriculture has become increasingly important and

the utilization of biofertilizers a feasible practice of production. Vessey (2003) proposed that biofertilizer be defined as a substance that contains living microorganisms which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant. Marketable biofertilizers are mainly based on *Rhizobium*, PGPRs, and AMF. Probably, the most ancient are those based on *Rhizobium*, which in addition to enhance yield, improve grain quality by increasing protein content and in some cases the lipid content (e.g., soybean) due to their nitrogen-fixing activity (Achakzai et al., 2003; Hayat and Ali, 2004). Not all the beneficial rhizosphere microorganisms exert their positive effect on plant growth via increasing nutrient status of host plants. PGPR are long known to promote growth through suppression of plant disease acting as bioprotectants (Kloepper, 1993), or through production of phytohormones and peptides acting as biostimulants (Glick et al., 1998; Jiménez-Delgadillo, 2004).

Although the beneficial influence of AMF on the growth of crops is mostly due to the better mineral nutrition, it is known that they protect plants against cultural and environmental stresses (Smith and Read, 1997). AMF-plant symbiosis can also alter plant water relations and responses to drought (Augé, 2001), photosynthesis rate (Aguilera-Gómez et al., 1999), and provide host plant with growth hormones like auxins, cytokinins, gibberellins, and growth regulators such vitamin B (Gupta et al., 2000).

Product quality as determined by the ultimate consumer is affected by both pre- and postharvest factors (Schreiner et al., 2000). A wide variety of preharvest factors influence the quality of the harvested product. These include (1) biological factors (pathological, entomological, animal); (2) physiological factors (physiological disorders, nutritional imbalances, maturity); (3) environmental (e.g., climate, weather, soils, water relations, and light intensity), cultural factors (fertilization, growth regulators); (4) extraneous matter (growing medium, vegetable matter, chemical residues); and (6) genetic (cultivars, aberrations) (Kays, 1999; Mattheis and Fellman, 1999; Sams, 1999). It is evident that a diverse range of biotic and abiotic factors can alter agricultural product quality. We propose rhizosphere microorganisms to be considered as preharvest biotic factors affecting fruit and vegetable quality. This is why some interactions in the rhizosphere are of current concern and their effects on crop yield and quality are described and discussed here.

17.2 Plant growth–promoting rhizobacteria

Rhizobacteria that exert beneficial effects on plant development are referred to as plant growth–promoting rhizobacteria (Kloepper et al., 1980), because their application is often associated with increased rates of plant growth. PGPR enhance plant growth by direct and indirect means, but the specific mechanisms involved have not been well characterized (Glick et al., 1998).

With regard to the indirect mechanisms for growth promotion, one of the most important is biological control, and consequently the applied work with PGPR has mainly been based on their role in biological control. Bacteria in the genera *Streptomyces*, *Burkholderia*, *Agrobacterium*, *Pseudomonas*, and *Bacillus* are the most studied and commercialized biological control agents. They suppress plant disease through at least one mechanism: induction of systemic resistance, antibiotic production, depletion of iron from rhizosphere,

synthesis of antifungal metabolites, the production of fungal cell wall–lysing enzymes or competition for sites on the root (Glick et al., 1998).

Regarding the direct mechanism of facilitating plant nutrition, the means by which PGPR enhance the nutrient status of host plants can be categorized into five areas: (1) biological nitrogen fixation; (2) increasing the availability of nutrients in the rhizosphere; (3) inducing increases in root surface area; (4) enhancing other beneficial symbioses of the host; and (5) combination of modes of action (for a review, see Vessey, 2003). Examples of PGPR that exert a positive effect on plant growth–facilitating nutrients uptake are nitrogen-fixing bacteria (*Azospirillium*), siderophore-producing bacteria (*Pseudomonas*), sulfur-oxidizing bacteria (*Thiobacillus*), and phosphate-mineral solubilizing bacteria (*Bacillus*, *Pseudomonas*) (Vessey, 2003).

The most well-studied PGPR are the rhizobia (including the *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium*) for their ability to fix nitrogen in their legume hosts. The role of biological nitrogen fixation as a mechanism for growth promotion is still controversial. Many biofertilizing PGPR produce phytohormones, which are believed to modify growth patterns in roots by changing assimilate-partitioning patterns in plants. Those modifications might increase the absorptive surface of plant roots for uptake of water and nutrients. There are species of *Pseudomonas* and *Bacillus* with the ability to produce phytohormones (e.g., indole acetic acid, cytokinins, and gibberellins) and growth regulators. However, only in the cases of *Pseudomonas putida* (Hall et al., 1996), *Bacillus subtilis* (Jiménez-Delgadillo, 2004), and *Azospirillum*, direct evidences exist for a role of plant growth regulators in PGPR-elicited growth promotion. In fact, the most well-studied PGPR system in nonlegume hosts is the nitrogen-fixing genus *Azospirrillum*. Inoculation of seeds with nearly all *Azospirillum* strains causes increases in root length, number of root hairs, number of root branches, and root surface area (Bashan and Holguin, 1997, 1998). Different mechanisms, such as phytohormone production, nitrate reduction, and nitrogen fixation, have been proposed to explain improved plant growth following inoculation with *Azospirillum*. The production of indole-3-acetic acid (IAA) by *Azospirillum* appears to be the most likely explanation for growth promotion. However, it is highly unlikely that IAA alone causes yield increases that have been reported on a large number of crops (reviewed by Bashan and de-Bashan, 2004).

The effect of PGPR-promoting root lengthening has been explained by Glick and coworkers (Hall et al., 1996; Glick et al., 1998). The proposed model suggests that bacterial production of IAA stimulates plant cell proliferation or elongation and results in plant production of 1-aminocyclopropane-1-carboxylate (ACC), an ethylene precursor. The ACC produced by the host plants is taken up by the PGPR *P. putida* strain and is cleaved by ACC deaminase, resulting in a decrease of ethylene production in roots. The net biological effects of this system are increased root elongation of the plant and the nitrogen source for the PGPR. The more recent discoveries of the involvement of cytokinins (de Salamone et al., 2001) and possibly gibberellins (Gutierrez-Manero et al., 2001) opens the possibility that even more plant growth–regulating substances may be involved in the promotion of plant growth by some PGPR. Undoubtedly, more plant growth–regulating substances have yet to be discovered. It is likely that the mode of action of currently identified and yet to be discovered PGPR will involve production of substances, which will mimic or influence the action of these newer plant growth–regulating substances (Vessey, 2003).

Research on mechanisms of growth promotion has shown the complexity of PGPR–plant association. Currently, the role of active volatiles other than ethylene produced by PGPR has been added to that complexity. Ryu et al. (2003) reported that the volatile compounds 2,3-butanediol and acetoin, produced by *B. subtilis* and *Bacillus amyloliquefaciens*, promoted growth of *Arabidopsis thaliana*. In this sense, most of our work has been done with systems based on *Bacillus* spp. showing that growth promotion is also related with the production of peptides (Jiménez-Delgadillo, 2004).

17.3 Arbuscular mycorrhizal fungi

As reported in the literature, a variety of mechanisms have been proposed for how AMF may exert positive effects on plant physiology. The colonization of plant roots by AMF can greatly affect the plant uptake of mineral nutrients. The contribution of AMF to plant nutrient uptake is mainly due to acquisition of nutrients by the extraradical hyphae (George, 2000). The plant makes use of the tremendous surface area of mycelium to absorb mineral nutrients from the soil. It is also believed that the mycelia of mycorrhizal networks have chemical absorption capabilities that are able to access some plant nutrients at lower concentrations that nonmycorrhizal plant roots are not capable of absorbing. Many arbuscular mycorrhizal fungi can transport nitrogen, phosphorous, zinc, and copper to the host plant. It has been shown that phosphate in soil solution is absorbed by a phosphate transporter in the extraradical hyphae, condensed into polyphosphate and translocated through the intraradical hyphae, being released across the fungal membrane in the arbuscule (Saito, 2000). The plant water balance could also be affected by AMF. The mycorrhizal influence most often examined within this field has been the alteration of stomatal behavior in host plants. Moreover, mycorrhizal symbiosis affects photosynthesis, a physiological process intimately linked with stomatal behavior (Augé, 2001).

It has been suggested that phytohormones released by the infecting fungi may contribute to the enhancement of plant growth, since elevated hormonal levels in mycorrhizal plants have been reported (Ludwig-Müller, 2000). Although it has been shown that mycorrhizae play a significant role somehow protecting plants against phytopathogenic organisms, the experimental demonstration of this phenomenon has been difficult. However, different mechanisms have been suggested: (a) enhanced nutrition, (b) competition for nutrients and infection sites, (c) morphological changes, (d) changes in chemical constituents in plant tissues, (e) alleviation of abiotic stress, and (f) interactions with other rhizosphere microorganisms, occurring in the zone of soil surrounding the roots and fungal hyphae commonly referred to as the "mycorrhizosphere" (Rambelli, 1973). It is a fact that the success of root colonization and the mycorrhizal effectiveness depend on a variety of biotic and abiotic factors including the species of arbuscular mycorrhizal fungi forming the symbiosis, plant cultivar, availability of P in soil, and type of soil, among others.

The complex cellular relationship between host roots and AMF requires a continuous exchange of signals, which in return affects the regulation of genes whose products participate in metabolic and structural changes that lead to the symbiosis. AMF colonization induced genes related to defense or stress responses, and they showed an increase in transcript level during the initial period of contact between the symbionts with a subsequent decrease as the symbiosis developed (Liu et al., 2003). Transcription of new genes that are similar to the components of signal transduction pathways had also shown a sustained increase,

which correlated with the colonization of the root system and proliferation of the fungus within the roots (Gianinazzi-Pearson et al., 2000; Liu et al., 2003). Regarding biochemical and physiological changes found in mycorrhizal plants, it has been shown that specific proteins (Gianinazzi-Pearson and Gianinazzi, 1989), amino acid fractions (Tawaraya et al., 1994; Tawaraya and Saito, 1994), lipids (Bethlenfalvay et al., 1994, 1997), reducing sugars, and secondary metabolites are produced by host plants in response to AMF root colonization.

Interactions between PGPR and AMF have potentially beneficial functions, together with the majority of those where PGPR (Meyer and Linderman, 1986; Kloepper, 1994, 1996) including nitrogen-fixing bacteria (Biró et al., 2000) are involved. There is little information regarding the mechanisms controlling interactions of bacteria with AMF and plant roots in the mycorrhizosphere; however, a number of possible alternatives have been proposed. Some bacteria have been shown to directly affect AMF germination and growth rate (Carpenter-Boggs et al., 1995); thus, the beneficial impact to the plant could be through the mycorrhizal association.

17.4 Changes in product quality due to crop inoculation with beneficial rhizosphere microorganisms

Appearance is utilized throughout the production chain as the primary means of judging the quality of individual units of product. Product appearance is characterized mainly by the absence of defects, size, shape, and color (Kays, 1999). The introduction of arbuscular mycorrhizal fungi and plant growth–promoting rhizobacteria for crop production not only increases total yield, but also has a positive effect on appearance resulting in greater marketable yields.

17.4.1 Marketable quality

Field and greenhouse experiments have shown increased yields and enhanced quality of fruit and vegetables resulting from PGPR and AMF inoculation. By adapting such methods of production, homogeneous produce quality is obtained at the time of harvest. Additionally, we have observed that products belonging to the first harvest keep the best quality compared to that obtained with conventional production methods.

Table 17.1 shows the results of a field experiment carried out in Jalisco, México. Bell pepper fruits produced through the conventional method were compared to those produced by inoculating plants with commercial arbuscular mycorrhizal inoculants. Fruits were classified into four different quality categories following criteria of marketability (unpublished

Table 17.1 Effect of the arbuscular mycorrhizal fungi bell pepper fruit quality

Treatments	First harvest	Second harvest	Third harvest
Conventional production	R	G	R
Tec-Myc	E	E	G
Endospore	E	G	R

Commercial inoculants, Tec-Myc and endospore; E, excellent quality; G, good quality; R, regular quality; L, low quality.

Table 17.2 Effect of the arbuscular mycorrhizal fungus *G. fasciculatum* on yield and fruit weight of tomato (*L. esculentum* Mill. cv. Río Fuego)

Treatments	Yield/plant (g)	Marketable grade yield (%)	Weight/fruit (g)
CTL	622b	76	67.8b
Gf	963a	84	74.6a

CTL, noninoculated control plants supplied with full strength P (44 mg/L).
Gf,*G. fasciculatum*-inoculated plants supplied with low P (22 mg/L).
Means of yield followed by different letter are significantly different based on minimum significant difference (MSD) test ($p < 0.05$, $n = 4$). Means of weight followed by different letter are significantly different based on MSD test ($p < 0.05$, $n = 10$).

data). In this sense, Vavrina (1999) found differences in pepper quality after inoculating the plants with different PGPR strains. In general, fruits of better quality as well as greater number of extra large and large fruits were obtained from inoculated plants compared to those from plants cultivated by conventional fertilization. In a similar way, inoculation of tomato (*Lycopersicon esculentum* Mill.) with either AMF or PGPR leads to increasing total and marketable yields as a consequence of improving appearance, particularly size (Tables 17.2 and 17.3).

Potato (*Solanum tuberosum* L.) also responds positively to the AMF inoculation. Duffy and Cassells (2000) reported that arbuscular mycorrhizal inoculants improve economic yield quality in potato micropropagation. The average number of seed grade tubers in plants inoculated with a commercial mixture of isolates resulted 2.2 times higher than that found in noninoculated microplants. Their study also revealed the dependence on the mycorrhizal isolate and the host genotype, which pointed to the need for more investigation in different potato cultivars. As well, it is widely known that AMF help host plants to tolerate water stress and those benefits are reflected in the quality of fruits grown under stress conditions. In this sense, Kaya et al. (2003) reported that mycorrhizal inoculation with *Glomus clarum* significantly reduced the detrimental effect of water stress on fruit yield of watermelon (*Citrullus lanatus* Thunb.) with higher total yields (35.26 kg/plant). Marketable fruit yield was at its lowest in the water stress noninoculated plants (17.60 kg/plant), while AMF in water-stressed plants restored fruit yield (26.13 kg/plant) to a level similar to well-watered plants (27.96 kg/plant).

Table 17.3 Effect of the growth-promoting rhizobacteria *B. subtillis BEB-13bs* on yield and fruit weight of tomato (*L. esculentum* Mill. cv. Río Fuego)

Treatments	Yield/plant (g)	Marketable grade yield (%)
Noninoculated plants	648b	66
BEB-13bs-inoculated plants	787a	72

Means of yield followed by different letter are significantly different based on MSD test ($p < 0.05$, $n = 4$). Means of weight followed by different letter are significantly different based on MSD test ($P < 0.05$, $n = 10$).

The alleviation of water stress manifested by reaching acceptable fruit quality was also reported by Mena-Violante et al. (2006). Under drought conditions, pepper plants inoculated with any of the two AMF consortia–produced fruits with fresh weights (41.3 and 43.9 g) similar to those of fruits in noninoculated plants not subjected to drought (40.8 g). Additionally, fruits from plants subjected to drought and inoculated with a consortium reached the same size (width 5.0 cm; length 7.8 cm; pedicle length 4.6 cm) than those in noninoculated plants not exposed to drought (width 4.8 cm; length 8.1 cm; pedicel length 4.4 cm). Thus, inoculation of plant with AMF consortia mitigated the detrimental effect of water stress on fruit fresh weight and size.

Quality of tropical fruits is also improved through PGPR inoculation. Baset Mia et al. (2005) demonstrated the positive effect of PGPR inoculation along with a reduction (67%) of recommended N fertilizer on banana yield and quality. The authors suggested that strains of *Azospirillum brasilense* and *Bacillus sphaericus* could be used for high yielding and improved physical attributes such as finger weight, length, diameter, and pulp/peel ratio. Moreover, we have performed field trials working together with local crop producers, where the inoculation with AMF and PGPR have brought increases in marketable yields in pepper, tomato, potato, strawberry, melon, and lettuce ranging from 15 to 35%, especially in the very first three harvests (V. Olalde-Portugal, unpublished data).

17.4.2 Shelf life

Improving produce quality at harvest is associated with beneficial changes in the keeping quality of the product during postharvest storage. It is desirable to achieve a longer shelf life of horticultural products, minimizing quality-reducing alterations such as water loss, drastic color changes, spoilage derived from pathogenic microorganism and softening, among others. Our greenhouse experiments showed that the inoculation of tomato plants with AMF enhanced tomato shelf life. The fruits of noninoculated plants decayed earlier than those of inoculated plants. Seventy percent of fruits produced by AMF plants were still marketable after 10 days of storage at 25°C, while only 33% of fruits from noninoculated plants were not decayed at that time (Mena-Violante et al., 2003).

Regarding the practice of inoculation and introduction of more than one fungus and/or bacterium into the target crops, we found that the AMF and PGPR inocula seem to have a synergistic effect on tomato shelf life. Figure 17.1 shows the percentages of marketable tomato fruits after 8, 15, 22, and 30 days of storage at room temperature. Tomato plants were managed by the conventional method using full doses of chemical fertilizers or using biological inoculants in combination with 25% of recommended P fertilizer. A higher percentage of fruits were marketable in inoculated plants than that presented in plants cultivated conventionally after the same storage time.

The efficient operation of the antioxidant enzyme system can result in a better quality produce with longer shelf life as a result of the maintenance of cellular structure and thereby the integrity of tissue. The levels of antioxidants such as ascorbate and vitamin E in the fruits may be indirectly influenced by the expression levels and activity of antioxidant enzymes such as superoxide dismutase, catalase, and ascorbate peroxidase (Ahn et al., 2002, 2005). Therefore, AMF could be involved not only in a better P uptake but also in the antioxidant levels in fruit. This could be an interesting research area to understand the enhanced shelf life derived from plant AMF inoculation.

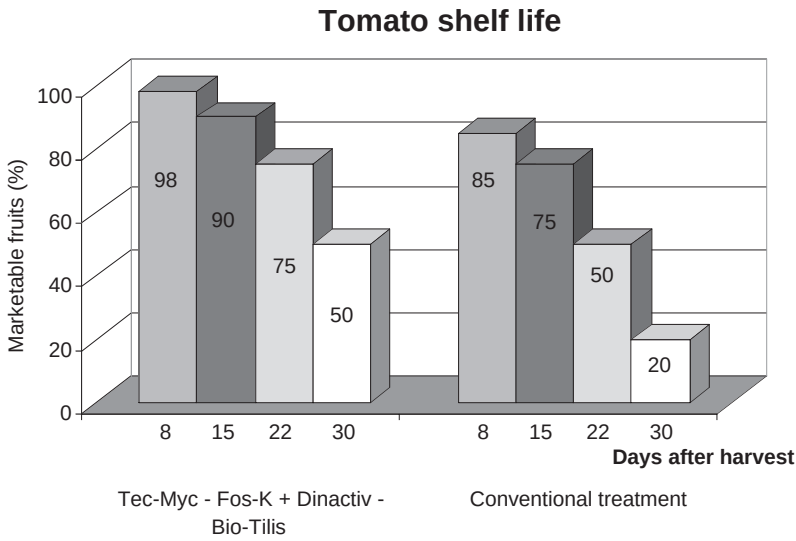

Fig. 17.1 Effect of plants inoculation with AMF and PGPR on tomato fruit shelf life. Tec-Myc, AMF-based inoculant; Bio-Tilis, *B. subtilis*–based inoculant; Dinactive, fertilizer; conventional treatment.

17.4.3 Composition

As mentioned before, microorganisms associated with plant roots cause biochemical and physiological changes in host plants. Such changes are mirrored in seed quality in terms of relative abundance of storage products such as proteins and lipids. For example, soybeans show an increased content of oil (5.3%) and protein (1.5%), while the content of soluble sugars and starch decrease (21.8 and 11.6%, respectively), when plant roots are inoculated with *Rhizobium* (Achakzai et al., 2003). Inoculant formulations improved the number of nodules, grain yield, seed protein concentration, and grain nitrogen content over the non-inoculated (including the N fertilizer) treatments. The most pronounced effect of AMF has been enhanced P nutrition of host plants (Kothari et al., 1991; Al-Karaki and Al-Raddad, 1997). In seeds, the relative abundance of storage products such as proteins and lipids is altered by plant inoculation with AMF (Bethlenfalvay et al., 1994; Al-Karaki and Clark, 1999). Bethlenfalvay et al. (1994) studying pea (*Pisum sativum* L.) concluded that the presence and intensity of AMF colonization altered the response of seed lipid metabolism in response to increasing P availability, which in turn affected the protein and lipid ratios. Thus, the plant P status can affect seed protein/lipid (Pro/L) balances (Bethlenfalvay et al., 1997). However, different relationships between seed lipid and protein contents in wheat (*Triticum durum* L.) plants inoculated or noninoculated with AMF indicated that the AMF effects on seed composition could be distinct from those mediated by P nutrition provided through conventional means (Al-Karaki and Clark, 1999).

It is known that organically grown produce possessed higher levels of antioxidants (Asami et al., 2003). In pepper fruit, vitamin C content is altered by root plant inoculation with AMF. Bagyaraj and Sreeramulu (1982) found that the ascorbic acid content of the green chilli was increased in inoculated plants (up to 11%) supplied with the half-recommended P. However, Oke et al. (2005) observed that phosphorus fertilizer application did not appear to stimulate the content of vitamin C in tomato fruits. Although it is possible that the beneficial

effects of AMF on fruit may be provided through enhanced phosphorus uptake among other factors, in our experiments the positive impact of AMF on fruit is present even at low P level fertilization; therefore, the role of P uptake in enhancing quality of fruits in mycorrhizal plants is not clear.

17.4.4 Secondary metabolite production

Interestingly, it has been shown that the production of essential oils could be influenced by AMF inoculation as well. Kapoor et al. (2002) suggested that the utilization of AMF may improve the quantitative and qualitative yield of essential oil in coriander (*Coriandrum sativum* L.) fruits. Fruits of plants inoculated with *Glomus macrocarpum* or *Glomus fasciculatum* contained more essential oil (28 and 43%, respectively) than those of noninoculated plants. Moreover, gas chromatography of essential oil showed increased concentration of geraniol and linalool in plants inoculated with *G. macrocarpum* or *G. fasciculatum*, respectively.

From the commercial point of view and due to the growing interest in the bioactive components, pigment content should be taken into account for evaluating the effect of AMF on product quality. Mena-Violante et al. (2006) found that plant inoculation with AMF consortia decreased chlorophyll concentration and increased carotenoids concentration in fruit of *Capsicum annuum*. Additionally, concentration of pigment was significantly influenced by the AMF inocula under drought. Thus, carotenoid concentration increased in fruits of plants inoculated either with *G. fasciculatum* or AMF consortium when compared to noninoculated plants not exposed to drought. Mena-Violante et al. (2006) suggested that the color changes found in fruits of *C. annuum* inoculated with AMF could be related to the ripening process due to the alterations in chlorophyll and carotenoid accumulation patterns. However, more work must be done to substantiate these results. It is worthy to point out that under drought, fruits in plants inoculated with *G. fasciculatum* reached the same C* values and chlorophyll concentrations of those in noninoculated plants not subjected to drought. Color attributes of tomato fruits from plants subjected to AMF inoculation are given in Table 17.4 (Mena-Violante et al., 2003).

17.4.5 Ripening

Fruit ripening is a highly complex process, characterized by a series of coordinated biochemical and physiological changes that lead to the development of a soft, edible fruit.

Table 17.4 Effect of the arbuscular mycorrhizal fungus *G. fasciculatum* on fruit color of tomato (*L. esculentum* Mill. cv. Río Fuego) at the red stage

Treatments	Phosphate (mg/L)	L^*	A^*
Noninoculated plants	22	21.0c	1.28c
	44	21.6c	2.18b
G. fasciculatum-inoculated plants	22	23.7a	5.66a
	44	22.3b	2.36b

L^* indicates lightness from white = 100 to black = 0.
A^* is the red-green axis (from −60 to +60).
Means followed by different letter within each column are significantly different based on MSD test ($p < 0.05$, $n = 10$).

Such changes influence the biosynthesis and deposition of carotenoids in chromoplasts the degree of which determines the intensity of color development, the metabolism of sugars and acids involved in flavor, and modifications to the structure and composition of the cell walls affecting fruit texture (Hobson and Grierson, 1993). Thus, ripening influences sensory attributes of fruit quality, such as flavor, appearance (shape, size, and color), and texture.

The main cause that reduces fruit quality is an excessive softening that influences shipping, storage, and market value (Giovannoni, 2001). Therefore, textural quality is one of the most important fruit properties to take into account along the production chain. It has been shown that AMF inoculation has a positive effect on texture; for example, bulbs of onion plants inoculated with *Glomus versiforme* are firmer than those inoculated with *Glomus intraradices* (Charron et al., 2001), which may be due to an increase in cell wall calcium content. In tomato, fruits of plants inoculated with *G. fasciculatum* showed a penetration force 1.2 times higher than those in noninoculated plants, when evaluated by a puncture test at different ripening stages, indicating that AMF improve fruit texture (Mena-Violante et al., 2003). Not only plant inoculation with AMF enhances tomato fruit texture, but also the use of PGPR. Tomatoes from plants inoculated with *B. subtilis* were firmer than those from noninoculated plants (Mena-Violante and Olalde-Portugal, 2007).

It is well known that the modifications of the structure and composition of the cell walls affect fruit texture (Hobson and Grierson, 1993). These changes are due to the expression of genes, such as 1-aminocyclopropane-1-carboxylate synthase gene (ACS) and 1-aminocyclopropane-1-carboxylate oxidase gene (ACO), involved in the ethylene biosynthesis (Yang and Hoffman, 1984), and polygalacturonase gene (PG) related to cell wall breakdown (DellaPena et al., 1986), among others. Thus, to look more closely at the mycorrhizal effect on the fruit, mRNA levels of PG and ACO were examined. A decrease in the abundance of the transcripts of the both genes was observed when compared to that in fruits of noninoculated plants (Mena-Violante et al., 2003).

All these results have revealed that the symbioses between microorganisms and plants seem to be more complex than it was suspected. Given the role of PGPR and AMF influencing crop quality, the question arises how quality can be enhanced. Further investigation should be conducted to understand the basis of the variation in the quality of horticultural products, in order to improve prediction of commodity behavior and ensuring high quality.

17.5 Conclusions

Due to a greater health consciousness among the public, an increasing demand for high-quality products necessitates the development of novel technologies using mycorrhizal organisms for enhancing produce quality and shelf life to satisfy the consumers. Moreover, because of current public concerns about the environment, more attention is now being given to the organic production of fruits and vegetables in response to the growing goal to reduce nitrogen and phosphate fertilization levels in agriculture. Alteration of product characteristics due to preharvest factors strongly determines postharvest quality and the consumer satisfaction. The results presented here show changes of quality attributes with respect to preharvest biotic factors such the inoculation of plant roots with PGPR or AMF. Using such information, new strategies and technologies such as the use of biofertilizers could be applied to agricultural practices to improve product quality and build environmentally friendly agrosystems.

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Chapter 18

Biotechnological Approaches to Enhancing Tropical Fruit Quality

Miguel Angel Gómez Lim

18.1 Introduction

Most fleshy fruits exhibit a high metabolic activity when compared to other plant-derived foods such as seeds. This metabolic activity continues during postharvest storage and makes most fruit highly perishable commodities with short shelf life. It is estimated that around 50% of all fresh fruits and vegetables are lost due to such spoilage, but the exact figure is difficult to determine (FAO, 2006). Fruit quality parameters such as deficiency in flavor and aroma development, reduced shelf life, rapid softening and spoilage, sensitivity to low temperatures, and increased susceptibility to pathogen infection are all a consequence of postharvest deterioration and are major constraints to the availability of fruits. Postharvest problems have been partially solved for many commercial crops (particularly those of temperate climate) by harvesting them at the mature or green stage or by applying various physical and chemical treatments to the ripe fruits and storing them at low temperatures or in controlled atmospheres. However, many fruits, particularly tropical fruits, cannot be handled successfully using these methods. Many tropical fruits harvested at full maturity do not store well, but if harvested at the immature stage, they fail to ripen adequately. In addition, they are very susceptible to low temperatures. Tropical fruits are important in the diet of people in less developed countries, and are increasingly important as exports from many of these countries.

Genetic improvement of tropical fruit crops has a range of objectives, including generation of cultivars with (a) tolerance of biotic and abiotic stresses, with reduced size and altered shape (apical dominance) to increase orchard plant density, lower harvesting and pruning costs, shorten the unproductive period, and improve availability of radiation at the canopy, (b) simultaneous ripening for mechanical harvesting, (c) reduced juvenility period, and (d) higher nutritional value (sugar, oil, vitamins, flavonoids, etc.) to improve the organoleptic qualities and shelf life of fruits.

Conventional breeding of perennial tropical fruit cultivars has been limited by their long juvenile period (up to 20 years), low fertility, high levels of heterozygosity, various levels of ploidy, polyembryony, complex intraspecific incompatibility relationships, and severe inbreeding depression (Gómez Lim and Litz, 2004). Genetic diversity within many tropical fruit crop species is unexplored, and most cultivars are either seedlings from uncontrolled pollinations or dooryard selections. The production of many tropical fruit crops

is based on a rather limited number of cultivars, which are poorly characterized for genetic traits.

Molecular and biotechnological approaches involving genetic transformation, which is the main subject of this chapter, offer an attractive alternative to conventional genetic improvement. In what follows, a description will be made of the different attempts of application of biotechnology for improvement of tropical fruit.

18.2 Fruit ripening

Fruit ripening is a highly complex process, with marked variations in metabolism occurring between different types of fruits. Nevertheless, the process is characterized by a series of coordinated biochemical and physiological changes that lead to the development of (in many cases) a soft, edible fruit (Giovannoni, 2004). Some of these changes include synthesis of secondary metabolites associated with flavor and aroma, synthesis of pigments, degradation of chlorophyll, alterations in organic acids and cell wall metabolism, and a softening of the fruit tissue. At the molecular level, there are a large number of tightly regulated genes involved in specific processes in a highly coordinated manner (Giovannoni, 2004).

In general, fruits are classified as climacteric or nonclimacteric depending on their patterns of respiration and ethylene synthesis during ripening. Climacteric fruits are characterized by an increased respiration rate at an early stage in the ripening process accompanied by autocatalytic ethylene production. Many of the economically important fruit crops are climacteric, and therefore a large amount of research has been devoted to studying the biochemical and molecular pathways operating during the climacteric ripening of fruits. Nonclimacteric fruits, on the other hand, show a different respiratory pattern and display a lack of autocatalytic ethylene synthesis. Research in nonclimacteric fruit has been traditionally lagging behind climacteric fruit, and although there is considerable information, a clear picture of the mechanisms governing the ripening process in this class of fruit is still missing (Adams-Phillips et al., 2004).

Most of the research aimed at modifying ripening has centered on manipulation of fruit firmness (membrane and cell wall properties) and ripening rate (ethylene production or perception) in climacteric fruit. These two aspects are discussed at length in other chapters of this book and will not be discussed here. Instead, the discussion will focus on approaches for improving fruit quality.

18.3 Genetic transformation of tropical crops

Most of tropical fruit crops are perennial trees and, because of the limitations described above, genetic transformation seems to be the only practical solution for improvement of specific horticultural traits (Gómez Lim and Litz, 2004). Genetic transformation provides the means for modifying single horticultural traits without altering the phenotype. This capability is particularly valuable for perennial plants and tree species in which development of new cultivars is hampered by their long generation time. Targeting specific gene traits is predicated on the ability to regenerate elite selections of what are generally trees from cell and tissue cultures. The integrity of the clone would thereby remain unchanged except for the altered trait. However, the difficulty in regenerating many tree species from elite or mature-phase selections is one of the most serious obstacles for applying gene transfer

technologies to these plants (Petri and Burgos, 2005). In addition, very few genotypes of a particular species have been transformed and, in many instances, these genotypes are not commercially important (Petri and Burgos, 2005). With the tools available, it is currently possible to genetically alter practically any gene, as long as a suitable molecular probe is available, and analyze the function during fruit ripening. The ability to apply this technology to various fruits is expanding as the number of fruit-bearing plants that can be transformed and regenerated is also increasing. Currently, the list includes apple, muskmelon, papaya, strawberry, banana, raspberry, mango, avocado, and other tropical and subtropical crops species (Gómez Lim and Litz, 2004).

Evaluating the performance of transgenic fruit tree cultivars requires approximately 12 years or until fruiting and flowering have been observed, depending on the species (Gómez Lim and Litz, 2004). Thus, molecular breeding represents a highly efficient approach for developing improved, perennial fruit cultivars. At present, insertion of foreign genes into plant DNA occurs in a random fashion, which may lead to accidental inactivation of nontarget genes and to variable and unpredictable expression of the transgene itself and even to gene silencing (Kohli et al., 2003). The use of matrix attachment region (MAR) sequences has been proposed to minimize transgene silencing and uniformize transgene expression (Allen et al., 2000). MARs are DNA sequences that bind to the cell's proteinaceous nuclear matrix to form DNA loop domains. Transgenes flanked with MARs are thought to be able to form their own chromatic domain and thus be insulated from the influences of factors in the chromatin adjacent to its site of insertion (Hall et al., 1991). Because a large majority of plant chromatin is in an inactive conformation at any given time, insulating the transgenes with MARs may reduce the incidence of gene silencing and enhance transgene expression (Lorence and Verpoorte, 2004).

The usual approach is to produce a large of number of lines derived from independent transformation events and to select the best genotype among the transformants. A description of the current methods employed to transfer foreign genes to fruit crops is beyond the scope of this chapter, and the interested reader is referred to recently published reviews (Lorence and Verpoorte, 2004; Cotsaftis and Guiderdoni, 2005).

18.4 Metabolic processes related to fruit quality susceptible to manipulation by genetic transformation

In the past, traditional plant breeding centered on improving crop yield. However, consumers are increasingly paying more attention to product quality and composition. As an example, consumers prefer fruits with increased nutritional properties (vitamins, sugars, proteins, minerals, etc.) and ingredients that may help reduce the risk of certain cancer or cardiovascular disorders (e.g., lycopene and antioxidants). These requirements have increased the need for fruits with a longer shelf life and increased quality. In what follows, a discussion of different metabolic processes related to fruit quality that have been or can be genetically manipulated will be made.

18.4.1 Lipid metabolism

Even though they are not a main component of fruits, except avocado, lipids are actively metabolized during ripening, and they can even be employed as indicators of the progress

of the process. There seems to be a correlation between the pattern and timing of ripening in relation to the appearance of lipid peroxidation products, which involves free radical formation, with ethylene treatment significantly inducing their appearance (Meir et al., 1991).

Genetic manipulation of the lipid composition could potentially improve the nutritional value of fruits, the profile of aroma compounds, and the ability of plants to withstand low-temperature stress. At present, two strategies have been used to modify lipid composition in higher plants: (a) alteration of the major fatty acid level by suppressing or overexpressing a specific key enzyme in lipid biosynthesis and (b) synthesis of a fatty acid not found in the host plant. The identification of desaturases, enzymes that introduce a *cis*-double bond in saturated fatty acids, has led to the production of plants with an increased level of polyunsaturated fatty acids (Arondel et al., 1992) or increased chilling tolerance (Ishizaki-Nishizawa et al., 1996; Khodakovskaya et al., 2006). By suppression of oleate desaturase, the levels of oleic acid (C18:1) in transgenic soybean and of stearate (CI8:0) in transgenic canola were increased up to 80 and 30%, respectively (Baldoni and Rugini, 2001). An example of strategy (b) could be seen in canola that does not naturally produce laurate (C12:0), whereas transgenic canola contained laurate by introduction of the proper enzyme (Baldoni and Rugini, 2001).

In avocado, whose outstanding compositional feature is its high fat content, changes in lipids during ripening, including increases in the monoglyceride and free fatty acid fractions, probably result from degradation of triglycerides (Kikuta and Erickson, 1968). Lipid metabolism has been linked with color and flavor development of fruit crops during ripening. Storage lipids may be involved in some manner in the metabolic processes taking place during ripening (Seymour and Tucker, 1993). The majority of lipids found in many fleshy fruits are esters of long-chain fatty acids. An increased fatty acid oxidizing activity has been recorded in some fruits as ripening proceeds (Baqui et al., 1977). The level of total lipids does not normally change during ripening, but the concentration of individual fatty acids (particularly linoleic and oleic acids) may be altered in a particular manner depending on the fruit (Wade and Bishop, 1978; Meir et al., 1991). Products of β-oxidation are used in the synthesis of both carotenoids and terpenoid volatiles (Baker et al., 2006), which are important aroma components of many fruits. Interestingly, mRNA of one enzyme of the β-oxidation pathway, peroxisomal thiolase, has been found to be induced during mango fruit ripening (Bojórquez and Gómez Lim, 1995). This probably reflects an increased β-oxidation pathway activity during ripening whose products are important for aroma production. Recently, acyl CoA oxidase, the key enzyme of β-oxidation, has been identified and also found to be induced during fruit ripening (A. Nila and M.A, Gómez-Lim, unpublished results). Therefore, the role of these enzymes might be to metabolize fatty acids to produce volatiles compounds. This is an area barely explored.

The activity of alcohol: acyl CoA acetyl transferase has been correlated with fruit ripening and the production of aroma volatile compounds in fruits (Shalit et al., 2001). It is possible that the production of those compounds may be increased or modified by genetic engineering.

18.4.2 Cold tolerance

Many fruits are sensitive to low temperatures, particularly tropical products. However, many plants have the ability to increase freezing tolerance in response to low temperature, a

process known as cold acclimation (Thomashow, 1999). Plants use a wide array of proteins to protect themselves against low temperature and freezing conditions (Shinozaki et al., 2003). There have been many approaches aimed at inducing tolerance to low temperatures, based on traditional breeding as well as on horticultural practices and genetic manipulation. Two examples of the latter (the use of desaturases and the alternate oxidase) are discussed elsewhere in this chapter. Many cold-responsive genes have been identified, and some of them are also induced in response to other types of stress, which suggest that they belong to a family of genes responding to a stress signals in general. However, there also genes specifically involved in the response to low temperatures such as the small *CBF* gene family encoding transcription factors (Zarka et al., 2003). In *Arabidopsis*, the transcriptional factors CBF1, CBF2, and CBF3 (also referred to as DREB1b, DREB1c, and DREB1a, respectively) are rapidly induced by low temperature followed by expression of CBF-targeted genes, the CBF regulon, which acts to bring about an increase in freezing tolerance (Sharma et al., 2005). The genes induced by the CBF family contain the CCGAC core sequence also named C-repeat (Baker et al., 1994), which has been found to be essential for the low-temperature responsiveness of additional cold-induced plant genes, including the *Arabidopsis* gene *COR15A* (Baker et al., 1994), the *Brassica napus* gene *BN115* (Jiang et al., 1996), and the wheat gene *WCS120* (Ouellet et al., 1998).

Overexpression of CBF3 in *Arabidopsis* mimics the response of the plant during cold acclimation (Gilmour et al., 2000). The CBF1, 2, and 3 proteins, though highly similar in amino acid sequence, are not identical but they share redundant functional activities (Gilmour et al., 2004). Most of the work on *CBF* genes has been performed in *Arabidopsis*, and even though tomato contains three *CBF* homolog genes, tomato cannot cold acclimatize raising the question whether it has a functional CBF cold response pathway. Only the tomato *LeCBF1* gene, however, was found to be cold inducible, and constitutive overexpression of LeCBF1 in transgenic *Arabidopsis* plants induced the expression of *CBF*-targeted genes and increased freezing tolerance indicating that LeCBF1 encodes a functional homolog of the *Arabidopsis* CBF1-3 proteins (Zhang et al., 2004). However, constitutive overexpression of either LeCBF1 or AtCBF3 in transgenic tomato plants did not increase freezing tolerance (Zhang et al., 2004). It is concluded that tomato has a complete CBF cold response pathway, but that the tomato CBF regulon differs from that of *Arabidopsis* and appears to be considerably smaller and less diverse in function. It remains to be seen whether the other fruit crops contain the CBF regulon and whether it works as in *Arabidopsis*.

Antifreeze proteins are found in a wide range of overwintering plants where they inhibit the growth and recrystallization of ice that forms in intercellular spaces (Griffith and Yaish, 2004). Unlike antifreeze proteins found in fish and insects, plant antifreeze proteins have multiple, hydrophilic ice-binding domains. Surprisingly, antifreeze proteins from plants are homologous to pathogenesis-related proteins and also provide protection against psychrophilic pathogens (Sharma et al., 2005). Transferring single genes encoding antifreeze proteins to freezing-sensitive plants lowered their freezing temperatures by approximately 1°C (Breton et al., 2000). The identification of these freezing tolerance-associated proteins and the elucidation of their cryoprotective functions will have important applications in several fields (Atici and Nalbantoglu, 2003). Designing new strategies to improve cold tolerance in crop varieties could increase the plant productivity and also expand the area under cultivation.

One example of the above is grapevines. The fruit and wine industries need to maintain and potentially expand production despite increasing constraints in the form of pests, diseases, and temperature stress. However, this must be done without compromising the quality traits of the crop. Using the *CBF* genes described above, grapevines tolerant to low temperatures were recently generated (Fischer et al., 2004).

18.4.3 Protein metabolism

Research in this area has included the increase of essential amino acid content, the expression of storage protein genes in plant organs other than seeds, and the reduction of the content of allergenic proteins, by genetic manipulation. Work has been done, for example, on the transfer of genes-encoding proteins rich in essential amino acids (i.e., methionine and lysine) from other species. To improve the nutritional quality of soybean, a methionine-rich 2S albumin from the Brazil nut (*Betholletia excelsa*) has been introduced into transgenic soybeans (Nordlee et al., 1996). However, since the Brazil nut is a known allergenic food, the resultant transgenic soybean turned out to be allergenic as well. For that reason, methionine-rich proteins from sources not containing known allergens have been employed in similar experiments. A maize gene encoding the protein zein increased methionine content by over 80% in transgenic soybean seeds (Baldoni and Rugini, 2001). An amaranth (*Amaranthus hypochondriacus*) 11S globulin, one the most abundant storage proteins (amarantin) of the seed, was transferred into tropical maize (Rascón-Cruz et al., 2004). Total protein and essential amino acids of the best expressing maize lines increased 32 and 8–44%, respectively, compared to nontransformed plants. To increase the content of lysine in potato, Sevenier et al. (2002) employed two approaches: Introduction of a feedback-insensitive gene from *Escherichia coli* (lysCM4) involved in biosynthesis of the aspartate family of amino acids resulted in a sixfold increase of the lysine content, whereas introduction of a mutated form of the key plant enzyme of lysine biosynthesis (dihydrodipicolinate synthase) led to a 15-fold increase in lysine. Allergenicity could also be reduced by genetic transformation. The 14–16-kDa allergenic proteins from rice have been reduced by using the antisense technology (Tada et al., 1996) and the same strategies could be applied in fruit crops.

18.4.4 Flavor and carbohydrate metabolism

Considering the importance of flavor in fruits, it is surprising that no major advances have been made to identify flavor components and enzymes responsible for their biosynthesis in fruits. This might be a reflection of the complexity of this trait. Since many fruits contain high quantities of carbohydrates, particularly sucrose, genetic manipulation of the sucrose-metabolizing enzymes might provide a way to alter sugar content and, in turn, sweetness of fruit.

There are a large number of reports on the manipulation of different enzymes involved with carbohydrate metabolism. Transgenic plants have been produced containing invertase in the sense and antisense orientations (Roitsch and Gonzalez, 2004), antisense granule-bound starch synthase (Liu et al., 2003), antisense phosphorylase (Duwenig et al., 1997), sense and antisense ADP-glucose pyrophosphorylase (Rober et al., 1996; Weber et al., 2000), sense and antisense sucrose synthase (Fernie et al., 2002), antisense uridine diphosphate-glucose pyrophosphorylase (Zrenner et al., 1993), and sense and antisense

sucrose phosphate synthase (Worrell et al., 1991; Strand et al., 2000). All the resultant plants showed an altered carbohydrate content, and considering the potent effect, this process may have on plant metabolism, in general, the use of fruit-specific promoters to target the transgenes to fruit cells becomes imperative.

Another approach to enhance fruit flavor could be the use of a variety of sweet-tasting proteins, thaumatin, monellin, mabinlin, pentadin, brazzein, curculin, and miraculin (Faus, 2000; Sun et al., 2006). All of these proteins have been isolated from plants that grow in tropical rain forests. They elicit a sweet taste by binding specifically with taste receptors and are approximately 100,000× sweeter than sugar on a molar basis (Van der Wel and Ardvidson, 1978; Faus, 2000). The taste-modifying protein, miraculin, has the unusual property of being able to modify a sour taste into a sweet taste.

Transgenic plants have been generated containing monellin (in tomato) (Peñarrubia et al., 1992) and miraculin (in lettuce) (Sun et al., 2006), inducing sweet tasting phenotypes, and a thaumatin gene has been introduced into potato (Witty and Harvey, 1990), cucumber (Szwacka et al., 2002), pear (Lebedev et al., 2002), tomato (Bartoszewski et al., 2003), and apple (Dolgov et al., 2004). The main features of the transgenic fruits were a sweet taste and a liquorice aftertaste lasting for a few minutes. Interestingly, thaumatin has been shown to be strongly induced in ripening fruits (Clendennen and May, 1997; Tattersall et al., 1997).

There have also been attempts at looking for substitutes for the natural sweetener sucrose, and the low-molecular-weight fructans, polymers of fructose, have been suggested as an adequate replacement. These compounds resemble sucrose in their organoleptic properties, but are indigestible by humans. In addition, they cannot be used as a carbon source by caries-causing bacteria. To obtain high fructan plants, the gene encoding 1-sucrose—sucrose fructosyl transferase from *Helianthus tuberosus* was introduced into sugar beet (Sevenier et al., 2002). The transgenic plants showed a dramatic change in the nature of the accumulated sugar, 90% of the sucrose being converted into low-molecular-weight fructan.

18.4.5 Plant architecture

Modification of plant architecture by genetic manipulation is now a reality. This result has been achieved by overexpression of phytochromes. The phytochromes are a family of photoreceptors that function as photoreversible pigments in plants. By overexpressing phytochrome A, dramatic and beneficial effects have been obtained in transgenic rice plants such as significant reduction in plant height (which would facilitate harvesting), an increased number of panicles per plant (resulting in a 6–21% higher yield), early flowering, up to 30% more chlorophyll in the leaves (increased photosynthesis in the field), and finally the transgenic lines accumulated significantly more biomass per plant (Robson et al., 1996; Robson and Smith, 1997; Garg et al., 2005). Clearly, this is an area that ought to be explored further.

18.4.6 Flower formation

Flower development is an unpredictable and irregular process in many commercial crops. Applications of several chemicals are required to stimulate and coordinate the formation of flowers in several crops. Molecular genetic studies have shown that at least three classes of homeotic genes control the determination of floral meristems and organ identity in

higher plants (Krizek and Fletcher, 2005). Transgenic plants overexpressing genes such as *LEAFY* or *CONSTANS* have been generated (Putterill et al., 2004). These genes are sufficient to determine floral fate in lateral shoot meristems with the consequence that flower development is induced precociously.

As mentioned above, most tree species have a long juvenile period of at least 5 years, and the time to evaluate trees can be up to 20 years. This has hampered the development of new, improved cultivars by traditional plant breeding and poses a challenge for the generation of improved varieties when using plant tissue cultures techniques. Transgenic citrus plants overexpressing the genes *LEAFY* or *APETALA1*, which promote flower initiation in *Arabidopsis*, have been produced (Peña et al., 2001). Both types of transgenic citrus produced fertile flowers and fruits as early as the first year, and a shortening of their juvenile period was detectable. Furthermore, expression of APETALA1 was as efficient as LEAFY in the initiation of flowers, and did not produce any severe developmental abnormality. Both types of transgenic trees flowered in consecutive years, and their flowering response was under environmental control. In addition, zygotic and nucellar-derived transgenic seedlings had a very short juvenile phase and flowered in their first spring, demonstrating the stability and inheritance of this trait. These results have opened up new avenues for research in genetic improvement of fruit trees.

18.4.7 Color and pigment metabolism

The external color of fruit is an important factor in consumer preference. The principal pigments in many fruit are carotenoids and anthocyanins, which are synthesized via the terpenoid and phenylpropanoid pathways, respectively. Pigment synthesis manipulation by genetic means represents an interesting choice to modify the color of a fruit to make it more attractive to the consumer. In some cases, the goal would be to increase the color of the transgenic product. Color is particularly important in fruits to be employed for jams, marmalades, pastes, and even wine.

There have been a number of attempts to increase the content of carotenoids in fruit by genetic manipulation, not so much to alter the normal color of plant organs but for other reasons (discussed below), and as expected, many of these attempts have resulted in altered coloration of plant organs. However, there have been some examples of genetic manipulation of carotenoids to alter the color of plant organs. Phytoene synthase, an enzyme induced during fruit ripening, catalyzes the dimerization of two molecules of geranylgeranyl pyrophosphate to form phytoene, the first C40 carotene in the carotenoid synthesis pathway (Römer and Fraser, 2005). Expression of phytoene synthase in antisense in tomatoes produced pale yellow flowers and fruits that ripened to a yellow color (Bird et al., 1991). Lycopene could not be detected in those fruits, although other ripening processes such as polygalacturonase accumulation were unaffected. The carotenoid biosynthesis pathway has also been modified in tobacco plants using the *CrtO* gene from the alga *Haematococcus pluvialis*, encoding the β-carotene ketolase (Mann et al., 2000). Transgenic plants accumulated ketocarotenoids that changed the color of the nectary from yellow to red. The authors speculate that plant transformation with this gene may be used in the future to change the color of fruit.

Anthocyanins are flavonoid derivatives which are major secondary plant products well known for the blue, red, and purple coloration they provide to flowers, fruits, and leaves.

Flavonoids are derived from phenylalanine and acetyl CoA in a highly branched pathway leading to flavonols, flavanones, isoflavonoids, and anthocyanins (Forkmann and Martens, 2001). It is known that this complex pathway is regulated at the level of transcription of structural genes (Forkmann and Martens, 2001). In general, genetic manipulation of intermediate enzymes of the flavonoid pathway may change the final balance of these colored compounds and eventually the color of a given plant organ. For example, when a chalcone reductase gene from *Medicago sativa* was introduced in *Petunia*, the flavonoid biosynthesis pathway was redirected since neither chalcone reductase activity nor the product of the reaction, which was further transformed into a colored compound, is naturally present in *Petunia*, and the plant produced yellow flowers (Davies et al., 1998). Similar results have been obtained using different enzymes in other plants (Holton, 1995; Markham, 1996; Su and Hsu, 2003). This illustrates the complex equilibrium of the complete pathway and the difficulty of predictable effects after plant transformation with heterologous genes.

18.4.8 Parthenocarpy

The absence of seeds in fruits is a valuable trait, not only from the consumer standpoint, but because it may allow control of fruit development even under adverse environmental conditions for pollination and may be used in fruit crops to standardize and increase fruit size (Gorguet et al., 2005). Horticultural methods for inducing parthenocarpy include spraying of growth regulators, induction of genetic mutations, or modification of ploidy level (Bukovac and Nakagawa, 1967). The parthenocarpy trait is often polygenic and therefore more difficult to deal with in-breeding programs (Gorguet et al., 2005). Parthenocarpy development, in some fruits at least, may be triggered by a deregulation of the hormonal balance in some specific tissues, in particular, between auxins and gibberellins (Fos et al., 2000). An increased level of these hormones in the ovary can substitute for pollination and trigger fruit development. This has been convincingly demonstrated by genetic engineering when the *iaaM* gene, coding for the enzyme tryptophan monooxygenase, was introduced in tomato, tobacco, eggplant, strawberry, and raspberry resulting in parthenocarpic fruits (Rotino et al., 1997; Acciarri et al., 2002; Mezzetti et al., 2004). The *iaaM* gene converts tryptophan to indole acetamide, a precursor of indole acetic acid, and was driven by a placental ovule-specific promoter (DefH9). The expression of chimeric DefH9-*iaaM* starts during early flower development, and the construct mimics the hormonal effects of pollination and embryo development by increasing the content and/or the activity of auxin in the ovule.

Parthenocarpic fruits can also result by mutation of the *pistillata* gene (Yao et al., 2001) or by downregulation of the*sepallata* gene (Ampomah-Dwamena et al., 2002). Expression of the *Agrobacterium rhizogenes* rolB gene in the ovary can also induce parthenocarpy (Carmi et al., 2003). This is an equivalent approach to that of increasing the content of auxins in the ovary as rolB codes for a putative auxin receptor, which makes the plant more sensitive to auxins (Maurel et al., 1994). Finally, high-temperature stress can also result in a seedless phenotype (Young et al., 2004).

18.4.9 Nutritional value enhancement

Plants are the staple food for the vast majority of the world's population, but it is known that they may be deficient in essential nutrients. For that reason, there have been attempts

at increasing the content of various nutrients (vitamins, essential amino acids, flavonoids, lycopene, etc.) by genetic manipulation (Sevenier et al., 2002).

Vitamins are essential factors in the diet, and they must be obtained from the diet. In addition, some vitamins are used as functional additives in food products. The edible part of rice grains, the endosperm, lacks vitamin A, and a diet based mostly on rice consumption may eventually cause vitamin A deficiency (Tucker, 2003). An outstanding achievement has been the introduction of genes into rice that enabled the biosynthesis in the endosperm of β-carotene, the precursor of vitamin A (Ye et al., 2000). The grain of the transgenic rice had a yellow golden color and by itself contained sufficient β-carotene for human vitamin A requirements. The authors of this work have waived all intellectual property rights for exploitation of these technologies in the developing world, and are actively involved in assisting the International Rice Research Institute to breed stable and agronomically successful lines for use in vitamin A-deficient areas. Similar experiments have been performed successfully in rapeseed, where introduction of a phytoene synthase gene also increased the level of vitamin A precursor (Kishore and Shewmaker, 1999) and in tomato, where introduction of a bacterial phytoene desaturase increased the β-carotene content in fruits up to twofold (Römer et al., 2000).

Another lipid-soluble vitamin with an antioxidant role is vitamin E (α-tocopherol). Daily intake of this vitamin in excess of a recommended minimum is associated with decreased incidence of several diseases. Plant oils are the main source of dietary vitamin E, and they generally have a high content of the vitamin E precursor γ-tocopherol. Overexpression of γ-tocopherol methyl transferase greatly increased the seed level of α-tocopherol in *Arabidopsis* (Shintani and DellaPenna, 1998) and corn (Rocheford et al., 2002). Apart from these examples, transgenic plants containing elevated levels of vitamin C have also been produced (Herbers, 2003). These experiments have resulted in functional food with enhanced health benefits, but there are now many laboratories in the public and private sectors looking to achieve vitamin levels high enough in transgenic plants to merit extraction from the plant (Herbers, 2003). Attempts to increase the content of carotenoids in fruit by genetic manipulation are common (Romer and Fraser, 2005; Long et al., 2006), and the reason is because they posses potent antioxidative, photoprotectant, and anticancer properties (Fraser and Bramley, 2004; Hix et al., 2004).

Flavonoids are another group of secondary metabolites whose inclusion in the human diet, in particular the flavonol group (e.g., quercetin and kaempferol), may give protection against cancer and cardiovascular diseases (Chen et al., 1990; Hou, 2003; Ren et al., 2003). The biosynthetic pathway leading to the synthesis of these compounds has been known for a long time, and consequently, the design of strategies to increase the content of these compounds has been possible. For example, transformation of tomato with a gene from *Petunia*, encoding a chalcone isomerase or with the maize transcription factor genes *LC* and *C*1, has resulted in fruits with an increased content of flavonoids (Muir et al., 2001; Bovy et al., 2002). Interestingly, by suppression of an endogenous photomorphogenesis regulatory gene, *DET1*, by RNA interference technology both carotenoid and flavonoid contents were increased significantly, whereas other parameters of fruit quality were largely unchanged (Davuluri et al., 2005).

When vegetables are the major components in the diet, there is a certain risk of iron deficiency. Although some plants are rich in iron, availability is limited by the oxalic acid and phytate-like substances present in the plant, which may complex this element. Oral

administration of ferritin, a protein used by plants and animals to store iron, can treat anemia in rats. Consequently, soybean gene encoding ferritin, under the control of a seed-specific promoter, has been introduced into rice (Goto et al., 1999). Transgenic rice plants accumulated ferritin in the endosperm and up to threefold levels of iron in comparison to normal seeds. Interestingly, plants overexpressing ferritin appeared to be tolerant to oxidative damage and pathogens (Deak et al., 1999).

Lipids are also important components of the human diet. There is an increased preference for plant-derived oils to the animal fats because of health concerns. Plant oils are mostly used for human consumption as margarines, oils, and food ingredients. Triacylglycerols are the most important components of plant seed oils. Properties such as melting point, color, flavor, mouthfeel, spreadability, stability, and effects on human health are determined by the fatty acid composition of the triacylglycerols.

Nowadays, there is a trend toward a reduction of saturated fatty acids in the diet and an increase in unsaturated fatty acids. It has been known for many years that intake of monounsaturated fatty acids is associated with a lowered incidence of coronary artery disease (Keys et al., 1986). Therefore, the unsaturation of fatty acids and the increase of unsaturated fatty acids have been targets for modification by genetic engineering studies. The content of unsaturated fatty acids could be increased in soybean, maize, and canola and potentially in other crops by manipulating the expression of desaturase genes (Kinney et. al., 2002). There is now considerable evidence of the importance of n-3 long-chain polyunsaturated fatty acids in human health (Gill and Valivety, 1997). They are normally found in fish oils but plants can be genetically engineered to synthesize these important fatty acids as a sustainable alternative source (Napier and Sayanova, 2005).

18.4.10 Molecular farming

The production of plant-derived biopharmaceuticals is sometimes referred to as molecular farming. The word biopharmaceutical is applied to a naturally occurring or modified polypeptide, protein, DNA, or RNA product that is to be used for therapeutic, prophylactic, or in vivo diagnostic use in humans or animals. The main categories of biopharmaceutical products are proteins, antigens, therapeutic monoclonal antibodies, and polyclonal antibodies. The first report on the production of biopharmaceuticals in plants was published in 1992 (Mason et al., 1992); since then proof of concept has been well established and over 100 products have been expressed in plants, several clinical trials performed, and three plant-based biopharmaceuticals are already in the market (Streatfield et al., 2003; Woodard et al., 2003; Howard, 2004; Dus Santos and Wigdorovitz, 2005). Several cereals, and in particular maize, have been the system of choice for expression of antigenic proteins since the proteins can be expressed at high levels in the kernel and stored for prolonged periods without excessive deterioration (Streatfield et al., 2003). Plants are natural bioreactors, and potentially a cheap source of recombinant products (Fischer et al., 2004). However, one possible inconvenience of using plants as bioreactors for biopharmaceuticals is post-translational modifications introduced by the plant. It is known that plants can glycosylate heterologous proteins and attach a variety of carbohydrates, including some not present in animal cells (Faye et al., 2005). These extra carbohydrates can alter the properties of heterologous proteins. For that reason, a detailed analysis of the plant-based product is

imperative. Fruits are also ideal choices for production of biopharmaceuticals since this can be achieved in a totally contained atmosphere such as a greenhouse.

18.4.11 The alternate oxidase

The alternate oxidase is an enzyme involved in the cyanide-resistant respiratory pathway, and it transfers electrons from the ubiquinone pool to oxygen without energy conservation. The enzyme can use reductants (electron donors) that are produced in excess and cannot be used efficiently by the cytochrome pathway, preventing the formation of reactive oxygen species from an overreduced ubiquinone pool, and thus may be involved in acclimation to oxidative stresses (Umbach et al., 2005) and to low temperatures (Fiorani et al., 2005). In addition, the alternate oxidase may act as an important mitochondrial "survival protein" against programmed cell death (Robson and Vanlerberghe, 2002). It has also been studied in thermogenic species, and its activity correlated with heat production, necessary to volatilize foul-smelling compounds to attract insect pollinators. There is a significant participation of this pathway in the climacteric of many fruit. A cDNA coding for the mango alternate oxidase has been identified, and by northern blot analysis the message was detected in unripe fruit and shown to increase substantially in ripe fruit (Cruz Hernandez and Gómez Lim, 1995). These results showed, for the first time, the participation of this enzyme in fruit ripening at the molecular level and were subsequently confirmed by an independent group (Considine et al., 2001). The temperature in ripe mango pulp is up to 10°C higher than in unripe pulp, and this has been attributed to the activity of the alternate oxidase (Kumar et al., 1990). This extra heat might also serve to volatilize aroma-giving compounds. Unfortunately, no additional studies have been performed in other ripening fruit.

18.4.12 Genetic stability

Considering the time and effort invested in transferring a gene in long-lived perennials such as fruit trees, it is essential that stable patterns of gene expression are maintained for long periods of time. Although fruit trees are normally vegetatively propagated, the transgene should also expressed in the progeny. There have been several studies to address this issue both with marker genes (Vain et al., 2002; James et al., 2004) or with genes conferring novel agronomic traits, such as *rolABC* from *A. rhizogenes* in transgenic kiwi plants of staminate GTH and pistillate Hayward cultivars (Baldoni and Rugini, 2001). After 12 years, the staminate *rolABC* plants maintained the same morphology and the offspring (transgenic staminate X normal pistillate) was transgenic in 50% of plants. The cherry rootstock Colt, containing *RiT-DNA*, which seems to modify the scion vigour, showed stability after 4 years in the field (Baldoni and Rugini, 2001). R. Scorza and coworkers have performed extensive analyses on transgenic *Prunus domestica* carrying the plum pox virus coat protein (PPV-CP), *uidA* and *nptll* genes. Gene expression has been stable in the greenhouse for over 5 years and the progeny, produced from hybridization of transgenic plants carrying plum pox virus coat protein, expressed the transgenes (Ravelonandro et al., 1997). These results seemed predictable in the light of a study in *Arabidopsis* carried out to search for transcriptome changes associated with expression of transgenes regulated by constitutive promoters (El Ouakfaoui and Miki, 2005). Insertion and expression of the marker genes, *uidA* and *nptII*, did not induce changes to the expression patterns of the approximately 24,000 genes that

were screened under optimal growth conditions and under physiological stress imposed by low temperatures (El Ouakfaoui and Miki, 2005). This study showed that the transgenic and nontransgenic plants were equivalent in their global patterns of transcription, and it may contribute to the principle of substantial equivalence, which is used as a first step in the biosafety evaluation of transgenic crops.

Apparently, stability of transgenes in the genome of transformed plants depends on their correct physical integration into the host genome as well as on flanking target DNA sequences. The exact site of transgene insertion into a plant host genome cannot, at present, be controlled and is poorly understood. A detailed analysis of transgene integration in 19 independently derived transgenic barley lines was carried out by fluorescence in situ hybridization (Salvo-Garrido et al., 2004). The pattern of transgene integration appeared to be nonrandom, and there was evidence of clustering of independent transgene insertion events within the barley genome. The data from the transgene flanking regions indicated that transgene insertions were preferentially located in gene-rich areas of the genome.

In another study with different transgenic lines of aspen, inverse PCR analysis revealed an additional truncated T-DNA copy of 1,050 nucleotides adjacent to the left border of the complete copy in one of the lines (Kumar and Fladung, 2001). Sequencing of this truncated T-DNA revealed that it represented an inverted copy of part of the right half of the original construct, which would allow the inverted repeat to pair with right border sequences of the complete copy. This would explain the frequently observed reversion resulting in transgene loss due to intrachromosomal base-pairing leading to double-stranded loops of single-stranded DNA during mitotic cell divisions (Kumar and Fladung, 2001).

18.5 Future perspectives

The improvement of fruit crops has depended on various technologies that have had varying degrees of success. Conventional breeding has been very successful with herbaceous species, but improvement of perennial fruit crops by traditional means has been limited. Biotechnologies that could increase the efficiency of fruit crop improvement, in particular tropical crops, are, therefore, essential to generate improved cultivars with novel traits. For example, genetic mapping could provide breeders with the tools to make rapid progress in crop improvement. Functional genomics and proteomics could provide insights into genetic regulation of plant function and novel means for isolating genes for manipulation in transgenic plants. Older biotechnologies, including somatic hybridization, in vitro mutation induction, and selection, have rarely been applied to tropical fruit species for crop improvement. There have been predictions that biotechnology will play a significant role in the twenty-first century (Cantor, 2000).

Even though transgenic plants with improved agronomic traits have already been produced in several fruit species, efforts have mainly focused on resistance to biotic stress and fruit ripening, while less work has been done on, for instance, altering growth rate or providing cold stress resistance. It is likely that the focus for development in the coming years will be on multiple gene introductions to increase output traits such as increased nutritional value, vitamin content, or improved flavor components. Obstacles still exist for some species in fundamental methodology, including gene transfer, genetic selection, and efficient protocols for regeneration. However, it seems possible to overcome these limitations following the recent contribution of a double regeneration system, which allows one

to obtain and maintain morphogenesis in calli for a long time in fruit species such as apple, cherry, and olive (Baldoni and Rugini, 2001).

Work with tropical crops lags far behind that with herbaceous species. Genetic transformation of perennial fruit crops has generally depended on embryogenic systems, and therefore regenerants of the woody species. Many species must pass through a period of juvenility before they can be properly evaluated. Two alternatives have been utilized to overcome this limitation: (1) invigorating plant material through grafting of mature buds onto juvenile stock plants (Cervera et al., 1998); (2) constitutive expression of either the *LEAFY* or *APETALA 1* genes from *Arabidopsis thaliana* to shorten the juvenile phase and promote precocious flowering (Peña et al., 2001). Both of these innovations could stimulate more transformation attempts with perennial species.

It would be advisable to use new selectable markers instead of the traditional ones and reduce the percentage of loss, which can be as high as 40% in many fruit crops such as apple, pear, banana, citrus, and grape (Gómez Lim and Litz, 2004). It would even be better to get rid of marker genes altogether and to employ one of the several methods available to generate marker-free transgenic plants (Ebinuma et al., 1997; Daniell et al., 2001; Huang et al., 2004; Wang et al., 2005). Clearly, these approaches would help improve public acceptance and perception of transgenic plants.

The major hindrances that have stymied genetic transformation studies with tropical/subtropical fruit, however, concern lack of regeneration protocols for elite (mature phase) selections and the relative absence of molecular studies. The latter reflects the state of the science in many developing countries where tropical fruit crops are grown on a large scale and the relative severity of production and postharvest problems of the crop. Biotechnology studies involving fruit crops everywhere are generally underfunded, and national and international agencies should perhaps consider more support for research with these plants.

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Chapter 19

Postharvest Factors Affecting Potato Quality and Storability

Sastry S. Jayanty

19.1 Introduction

Potato (*Solanum tuberosum* L.) is an important crop grown in the temperate, tropical, and subtropical regions of the world as a staple food because of its high nutritional value. It ranks fourth in annual production behind the cereal species rice (*Oryza sativa*), wheat (*Triticum aestivum*), and barley (*Hordeum vulgare*) and is one of the major vegetables consumed (Ross, 1986). Potato is the highest-yielding crop per hectare of arable ground. In North America and Europe, potatoes are consumed primarily in the form of processed food, where as in Southeast Asia and Latin America, it is mostly consumed fresh (FAO-STAT, 2005). The fall season production accounts for 70% of the total potato crop in the United States. The majority of the crop is stored for long term to meet the demands of the fresh market and processing industries. A proper postharvest storage situation is essential for a yearlong supply of potatoes to the consumer. Postharvest losses are mainly due to poor handling and improper storage. They typically account for 8–30% of the overall loss in the potato industry, and sometimes even greater losses are reported. Disease and sprouting are the main postharvest issues facing the potato storage industry to ensure yearlong supply of quality potatoes to the consumer. Minimizing these losses greatly improves profitability.

The potato tuber is a modified underground stem. Tubers are formed below the soil at the tips of lateral shoots called stolons. The terminal bud of the stolon undergoes dormancy and initiates tuberization through both cell division and cell elongation. During tuberization, starch and other nutrients are translocated to the tuber. A tuber may have up to 20 eyes/bud that are arranged spirally. The buds on the tuber, after a period of dormancy, can give rise to sprouts if conditions are favorable to allow propagation by vegetative amplification. The tuber is an important human food source and also represents seed stocks to produce a crop. Seed potatoes form an essential element in the total production cost of potatoes. This crop is more vulnerable to diseases and pests in storage because many of these are transmitted with seed tubers. Certified seed potatoes form an essential element in the potato production areas to minimize losses due to disease and pests.

Potatoes are rich in carbohydrates and are also a good source of minerals and vitamins. Recent low-carbohydrate diets and misconceptions of potato painted a bad picture about nutritional value of the potato. An average (5.3–6.0 oz) potato with the skin contains 18% of

potassium and 6% of iron recommended dietary allowance. Iron in a potato is more readily available because of high vitamin C (45%) and low in oxalates. Potatoes are also a good source of trace amounts of thiamin, riboflavin, folate, magnesium, phosphorous, iron, and zinc with natural fiber in the skins. A 6-oz potato can give 3 g of highly digestible, lysine-rich protein. Contrary to the popular notion that potatoes are fattening, a 6-oz potato offers less than 10% of the daily value of carbohydrates and complex carbohydrates, which are a great source of energy. Potatoes have also one of the highest overall antioxidant activity among vegetables (National Potato Council, 2006–2007).

The primary demand of the fresh market and the processing industries is to get high-quality potatoes over the long-storage periods. The chemical composition of the potato is very important, not only for storage but also for processing and consumption. Storage conditions vary significantly depending on which type of industry the potatoes are intended. Storage regimes play a significant role in tuber carbohydrate metabolism and thereby significantly affect the value of the potatoes depending on their intended use. Dry matter content, sugars, proteins, and the nitrogen compounds in tubers are important for the potato-processing industry. Potatoes contain a relatively low dry matter content that renders them perishable and sensitive to high and low temperatures during storage. The main issues in potato storage are to minimize sprouting, respiration, dehydration, and disease. Losses due to the above-mentioned factors can be minimized by designing storages with adequate ventilation, humidification, and temperature control. The important aspects for long-term storage are bringing down temperature in a timely systematic manner once tubers are harvested while allowing sufficient time for wound healing and proper temperature control during the holding period.

The three primary potato markets are processing, fresh market, and the certified seed industry. In the United States the processing industry consumes 63% of production, followed by the fresh market at 31%, and the certified seed industry at 6% (National Potato Council, 2006–2007). There is an increasing demand for better quality produce and improved postharvest handling practices. These measures can assist in satisfying some of these demands, as well as providing a greater window of time for marketing produce. The goal of this chapter is to bring together recent advances that have been made in understanding key concepts of tuber postharvest physiology and how that information is applicable in managing the crop in storage. Tuber dormancy, sprouting, and wound healing are very important processes that influence postharvest storage conditions. Recent developments in the above-mentioned processes at biochemical and molecular levels are discussed in the following pages. Current knowledge on cold-induced sweetening (CIS) and the role of sprout inhibitors on tuber quality are also discussed.

19.2 Wound healing/curing

The typical injuries associated with mechanical harvesting operations include cuts, bruising, and periderm loss. Potato tuber sensitivity to mechanical damage and the factors that control this process is well understood. Most of these damages can be reduced by changing harvester settings, avoiding harvest during cold weather (below 10°C), and allowing sufficient time between defoliation and harvest for skin set to occur. Vine killing (desiccation) is the standard method for promoting proper skin set and periderm maturation, which reduces skinning and bruising during harvest operations.

A good skin set is an indication of tuber maturity. A properly matured periderm helps in reducing bruise and is a major factor that determines harvest timing. Typically, 2–3 weeks after vine killing is required for tubers to have a mature periderm. A torque meter is often used to quantify the tuber resistance to skinning, and it is a measure of skin set in tubers (Lulai and Orr, 1993). Experiments on tuber resistance to skinning indicate that tensile strength of phellum has a minor role and changes little during the process of maturation (Lulai, 2002). The major factor in tuber resistance to skinning is the phellogen shear component. As periderm matures, the phellogen shear component also increases. The immature periderm is prone to skinning injury, which is due to phellogen single tangential plane of fracture. During development, these cells undergo changes resulting in physical strengthening known as skin set. Pavlista (2001) evaluated skin set on different cultivars using a torque meter by measuring resistance to skinning after vine killing. He concluded that resistance to torque exerted by the torque meter is an accurate measurement of skin set, and it can be used to determine optimum harvest conditions. McGee et al. (1985) found that the effect of maturity on wound healing is a temporary phenomenon and is not seen after storage.

19.2.1 Ideal conditions for wound healing

Potato tubers that are bruised due to harvest and grading operations are more prone to weight loss. This is due to evaporation of water from the wounded area. These are potential points of entry for microorganisms during storage. Wound healing usually occurs over a 2–3-week period after the tubers are placed in storage. The rate of healing is dependent on temperature. The viability of tubers in long-term storage is dependent on the effectiveness of the curing process. There is significant variability among different cultivars in terms of storability, which is largely attributed to effectiveness of the curing process (McGee et al., 1985).

Wound healing is essential because wounds are ideal entry points for pathogens that cause Fusarium dry rot and Pythium leak, and they further lead to loss of water and solutes (Spooner and Hammerschmidt, 1992). Wound healing takes place when temperatures are maintained at 15–20°C with 95% relative humidity. Temperatures less than 10°C significantly slow down the healing process. Following curing, potato tubers moved to low-temperature (5–10°C) chambers with high humidity for long-term storage.

During the curing process, potato tubers form new layers of cells that protect the internal living tissues from dehydration, injuries, and pathogens. These multiple layers of tissue called “cork” or “phellum” to protect damaged or injured tissue. Potato periderm or skin consists of three tissue types: phellum, phellogen, and phelloderm. Cork, or phellem, is the outermost layer that is formed by the phellogen (cork cambium). Cork tissue is programmed irreversibly to be dead at maturity. This phenomenon is also seen in secondary (mature) stems, roots, and tubers. Cork cells are impermeable due to the deposition of suberin and waxes. Cork also contains triterpenoids and phenylpropanoid derivatives, which may deter pathogen attack. Cork that is formed as a result of a wound is also called wound periderm.

19.2.2 Chemistry of wound healing

In potato, during the process of curing, new layers of cells that form wound periderm are deposited with polyphenolic (aromatic ligninlike domain) and polyaliphatic (cutinlike) compounds by a process described as suberization (Bernards, 2002). The suberized skin of

the potato tuber is the most studied example of cork formation (Sabba and Lulai, 2002). The biosynthesis of the linear long-chain compounds forming the aliphatic suberin domain and cork aromatic components share a common pathway with cutin and lignin to a certain extent (Boerjan et al., 2003). The aromatic domain is a polyphenolic substance mostly composed of hydroxycinnamic acid derivatives. This aromatic domain is believed to link the aliphatic domain to the cell wall (Kolattukudy, 1980). The biosynthetic pathway for hydroxycinnamic acid was proposed by Cottle and Kolattukudy (1982) and Bernards and Razem (2001). Peroxidase activity associated with suberization has been detected in the potato tuber (Bernards et al., 1999). Identification of metabolic enzymes involved in aliphatic domain biosynthesis is limited to isolation from potato tuber disks. Lotfy et al. (1994, 1995) hypothesized that ferulate acyltransferase is involved in linking the aromatic monomer ferulate to the aliphatic domain. Recently, Soler et al. (2007) used a comprehensive approach to identify genes involved in suberin biosynthesis by developing microarrays from expressed sequence tags (ESTs) obtained from the suppression subtractive hybridization method. Out of 236 ESTs analyzed, 135 sequences showed homology to unique genes playing a role in cork biosynthesis.

Using histological methods, Sabba and Lulai (2002) compared the process of maturation in native periderm and wound periderm. In the case of wound periderm, staining with ruthenium red and hydroxylamine–$FeCl_2$ revealed that cell walls lack the pectins that allow the formation of calcium pectate in native periderm to strengthen cell walls. Peroxidase staining is more prominent in native periderm. Schreiber et al. (2005) compared the efficiency of wound periderm with native periderm for peridermal transpiration, and concluded that wound periderm is not as efficient as native periderm and significant water loss can occur even after wound healing. The qualitative differences in chemical composition between the two periderms are very minute, but the water loss in native periderm is 5–10 times higher even after 1 month of suberization.

19.3 Dormancy

Length of dormancy is an economically important trait in potato tubers. Understanding the dormancy process and the ability to manipulate these processes are important to increase storability and the availability of potatoes throughout the year. Dormancy is often described as a resting stage where growth pauses and tubers will not sprout even when placed under ambient conditions favorable for growth (Burton, 1989). Dormancy is necessary for plant survival and development. Potatoes remain dormant for a set period of time after harvesting depending on the cultivar. Potato dormancy starts at tuber initiation because the apical bud of the stolon remains dormant during tuber development and enlargement (Burton, 1989). Potato tuber meristems are described as endodormant, where growth inhibition is due to the effect of biochemical factors within the tuber (Hemberg, 1985). Dormancy is regulated by variety of factors such as genotype, physiological age of the tuber, and plant hormones. Dormancy-related mechanisms are controlled by four plant hormones (abscisic acid (ABA), cytokinin, gibberellic acid (GA), and ethylene) along with environmental factors and photosynthesis (Suttle, 2000).

19.3.1 Status of molecular-level understanding on dormancy

Most of the recent knowledge on hormonal interaction and mechanism during dormancy comes from the model plant system *Arabidopsis*. ABA is a dormancy-inducing agent, as

opposed to cytokinins and GAs that are needed to terminate dormancy (Suttle and Haltstrand, 1994). Recently, receptors for both GA and ABA were identified and cloned from *Arabidopsis*. GID1 is a soluble protein receptor of GA and interacts with DELLA protein SLR1 resulting in degradation of SLR1 through the 26S proteasome pathway. Degradation of DELLA protein leads to altered responses to GA (Ueguchi-Tanaka et al., 2005). DELLA proteins are a family of negative regulators of GA responses. ABA receptor FCA is a plant-specific RNA-binding protein localized in the nucleus. FCA interacts with FY factor (FLOWERING LOCUS Y) and downregulates FLC (FLOWERING LOCUS C). ABA binding prevents formation of the active FCA-FY complex required for FLC repression (Razem et al., 2006).

Dormancy is regulated largely by both internal (hormones and sugar) and external signals (light and temperature). The molecular biology of endodormancy has been studied in potato, poplar (*Populus deltoids*) (Hsu et al., 2006), and grape (*Vitus vinifera*) (Or et al., 2000). GA and ABA play antagonistic roles in the regulation of dormancy. Moreover, the antagonistic relationship and the ratio between these two hormones may be responsible for regulation of the transition from dormancy to germination and sprouting in seeds (Razem et al., 2006). Distinct mechanisms of interaction between GA and ABA are utilized for different developmental decisions in plants, and these interactions may be organ specific (Weiss and Ori, 2007). Recent work by Achard et al. (2004, 2007) and Reyes and Chua (2007) showed the complex interaction between ABA and GA. These hormones act through a common mediator MYB33. MYB33 promotes ABA responses in seeds and GA responses in flowers. MicroRNA159 (miR159) induced by both GA and ABA targets MYB33.

19.3.2 Role of ABA

ABA is involved in suppressing α-amylase expression mediated by Ser/Thr protein kinase, PKABA1 (Gómez-Cadenas et al., 1999). ABA is known as a plant stress hormone induced during both cold and drought stress (Gilmour and Thomashow, 1991). Cold- or drought-induced ABA accumulation blocks further growth and development through the cyclin-dependent kinase inhibitor gene (ICK1) to prevent cell division in buds (Wang et al., 1997). Inhibitor studies with fluridone using microtuber system resulted in early sprouting. External application of ABA restored tuber dormancy, and establishes the role of ABA in induction and maintenance of tuber dormancy (Suttle and Haltstrand, 1994). Three of eight quantitative trait loci on dormancy also mapped to ABA levels, further confirming ABA's role in tuber dormancy (Claassens and Vreugdenhil, 2000). During postharvest storage, ABA levels decrease as the number of days increases (Suttle, 1995). Sorce et al. (1996) reported that during dormancy there is an increase in ABA levels in tuber eyes that decrease once the sprouting process is initiated. Recently, Destefano-Beltrán et al. (2006a) by using quantitative reverse transcriptase-polymerase chain reaction studied the expression levels of genes involved in both biosynthetic and catabolic pathways of ABA. As dormancy sets in, there is an increase in the biosynthesis of ABA. As time progresses and dormancy ends, degradative enzymes are more active to reduce the ABA concentration in tubers (Destefano-Beltrán et al., 2006b).

19.3.3 Role of GA

During cereal seed germination, GAs induce the transcription of α-amylases and other hydrolytic enzymes to hydrolyze starch and proteins, thereby supplying nutrients to the

developing embryo. Overexpressing the gibberellin biosynthetic gene GA 20-oxidase resulted in early sprouting in transgenic tubers (Carrera et al., 2000). Suttle (2004a) observed that there is no difference between endogenous GA levels in the tubers from the beginning to the end of the dormancy period. But at the end of dormancy during sprout growth, GAs are rapidly synthesized. KNOTTED-like homeodomain proteins are involved in meristimatic growth. The expression of KNAP2 (a KNOTTED-like gene) is upregulated during dormancy onset, but downregulated during the breaking of dormancy in apple (Brunel et al., 2002). However, overexpression of a KNOTTED-like gene in potato reduced the level of GA, resulting in dwarf plants (Faye et al., 2003).

Ethylene and ABA are involved in the senescence process and are also implicated in the induction of endodormancy in several plant systems (Fedoroff, 2002). The role of ethylene in endodormancy of potato microtubers is also known (Suttle, 1998a). Synergistic interactions among ethylene, ABA, and phytochrome have been studied in sorghum and duckweed (Finlayson et al., 1998; Weatherwax et al., 1998). Short-term ethylene treatment breaks dormancy where as continuous treatment of ethylene promotes dormancy (Rylski et al., 1974). Dormant microtubers when treated with ethylene inhibitors sprouted earlier than controls. This condition can be reversed with ethylene treatment (Suttle, 2004b). Phytochrome has long been known to be a key regulator of light responses in plants. Quantitative trait locus analysis in poplar trees for dormancy induction is mapped to a region of the chromosome that contains a phytochrome-encoding gene (Frewen et al., 2000).

19.3.4 Role of auxins

The concentration of auxins increases in tuber eyes before the onset of sprouting (Sorce et al., 2000). Both auxin and GA functions overlap in cell expansion and tissue differentiation in plants. Auxins positively affect GA signaling in two ways: by inducing the GA biosynthetic gene (GA20 oxidase) expression in pea and tobacco and by promoting degradation of DELLA proteins in *Arabidopsis* roots (Wolbang and Ross, 2001; Fu and Harberd, 2003; Nemhauser et al., 2006). Increase in auxin concentration before dormancy break may be upregulating the biosynthesis of GAs to facilitate sprouting in potato tubers.

19.3.5 Role of cytokinins

External application of cytokinins on dormant tubers breaks dormancy. The dormant buds or eyes in potato tubers can be stimulated by cytokinins to sprout as they promote cell division. Precocious sprouting was reported when the cytokinin biosynthesis gene expressed in potato tubers (Ooms and Lenton, 1985). Overexpression of the *Sho* gene from *Petunia hybrida* increased levels of cytokinins, which resulted in the loss of dormancy in tubers (Zubko et al., 2005). An increased level of cytokinins and *cis*-zeatin was observed before sprout initiation in tubers (Suttle, 1998b; Suttle and Banowetz, 2000). Suttle (2004a) suggested that increasing sensitivity to cytokinins with dormancy progression is a result of an increase in receptors for cytokinin perception and cytokinin signal transduction pathways. Cytokinins, along with auxins and GA, act as dormancy-terminating agents in potato tubers.

Apart from above-mentioned hormones, brassinosteroids (Korableva et al., 2002) and jasmonic acid (Abdala et al., 2000) have shown to be involved, but further research to understand their specific role in dormancy is needed. Recent successes using genomics-based

approaches to understand hormone action and control of crucial physiological processes such as cell division have opened up new avenues for research on dormancy in plants. The emerging interplay between bud dormancy status and cell division suggests that these two fundamental processes are probably regulated by common signaling pathways.

Natural dormancy is extended in potato by controlling two essential storage parameters: temperature and humidity. Major losses in storage are due to shrinkage, disease, and sprouting. The sprouting factor is covered as a separate topic in the following pages. Shrinkage is due to loss of water from the tuber. Potatoes, if stored at high temperature, will lose 7–10% of their weight over a 3-month period. If stored at lower humidities, potato tubers will lose more of weight—up to 20% at times. Most of the diseases affecting potatoes stop growing around 4–6°C. Temperatures above this can result in losses. If the tubers are clean and unbruised at harvest, problems that occur in storage are minimal. So it is important to harvest disease-free potatoes and handle them with care. Potatoes stored in light conditions will turn green within a week. This is due to accumulation of the alkaloid, solanin, in tubers. Green potatoes are not marketable. Potatoes should be stored in dark, humid chambers, maintaining low temperature to prevent sprouting.

19.4 Cold-induced sweetening

Management of temperature remains the most important factor affecting postharvest storage life and quality of vegetable crops. Low temperatures reduce the rate of biological reactions within the limits of tolerance of a crop. However, in addition to temperature control, reducing water loss and other preharvest influences also play an important role in extending the storage life and to maintain, improve, or delay the loss in quality of the product.

Storing tubers at low temperature help in reduce sprout growth and disease. However, it results in the accumulation of reducing sugars in the tubers stored for longer periods below 10°C. This phenomenon is called cold-induced sweetening (Sowokinos, 1990). Intermediate storage temperatures (10–12°C) prevent this sugar accumulation. Both storage temperature and physiological age of the tuber affect sugar accumulation (Hertog et al., 1997). At low temperatures, starch is converted to mainly sucrose and its monosaccharide derivatives glucose and fructose (Levitt, 1980). Some of these sugars act as osmoregulators and cryoprotectants to help in acclamatory processes of certain plant species in order to survive high altitude and harsh winters (Larcher, 2002; Stitt and Hurry, 2002).

Reducing sugars cause bitter taste and darken the color of potato chips when they are fried in oil at high temperatures. This is due to Maillard reaction, the product of which is not acceptable to the consumer. Maillard reaction is primarily a nonenzymatic reaction resulting in the formation of dark-colored adducts when a carbonyl group of a monosaccharide-reducing sugar reacts with the amino group of a free amino acid during the frying process of chips and french fries (Marquez and Anon, 1986; Rodriguez-Saona and Wrolstad, 1997). Darkening of potato chip color is primarily associated with glucose concentration in the tuber (Coleman et al., 1993). This is a serious problem for the processing industry (Dale and Bradshaw, 2003). A reducing sugar content of 0.025–0.030% on a fresh weight basis is the maximum concentration allowed in tubers used for chips (Sowokinos and Preston, 1988).

Recently, many publications have shown that the formation of a neurotoxic compound, acrylamide, occurs when fried at high temperatures. Acrylamide is formed as a result of the

amino acid asparagine reacting with reducing sugars during the frying process (Mottram et al., 2002; Stadler et al., 2002). It has been found that acrylamide levels are proportional to the amount of reducing sugar present in the tuber (Williams, 2005). Ishihara et al. (2006) showed that treating tuber slices with warm water before frying resulted in reduced levels of acrylamide. In terms of food safety and from a processing standpoint, reducing free sugar accumulation during storage is an important trait in potato tubers.

In cold-stressed tubers, the mechanism by which starch mobilization and accumulation of reducing sugars is not well understood. The major differences observed in carbohydrate metabolism between tubers maintained at 4 and 25°C in storage suggest that different temperatures can influence the enzymes and associated kinetic processes. Sowokinos (2001) suggested that sugar content in a tuber at any given time is determined by pathways involved in carbohydrate metabolism and catabolism such as starch synthesis, starch breakdown by glycolysis, hexogenesis, and mitochondrial respiratory pathways. Many investigators try to understand the biochemical basis of these pathways by suppressing genes, by putting gene in antisense orientation, or by over expressing the gene using constitutive promoters that lead to accumulation of reducing sugars in storage at low-temperature conditions. In the following paragraphs, important enzymatic steps and their contribution to CIS are discussed.

19.4.1 Acid invertases

Starch degradation in the amyloplasts can occur either by hydrolytic or by phosphorolytic processes. Products of starch degradation are exported to the cytosol in the form of hexose phosphates (hexose-P) via the glucose phosphate–phosphate translocator or as free sugars via the glucose and/or maltose transporters (Smith et al., 2005). Cytosolic sucrose phosphate synthase **(**SPS**)** converts starch products to sucrose (Krause et al., 1998). Subsequently, a proportion of the sucrose may be hydrolyzed to glucose and fructose by acid invertase (Greiner et al., 1999). Activity of the acid invertase located in cell walls and vacuoles was reported to be associated with the ratio between hexose and sucrose (Zrenner et al., 1996). Acid invertase in potato is susceptible to selective splicing induced by cold stress. This suggests that exon 2 could be the signal responding to the cold that causes an increase in transcription of acid invertase and hence a rapid degradation of sucrose and cold sweetening (Sturm, 1999). Hajirezaei et al. (2003) showed that cytosolic invertase could block the phloem transport of sucrose resulting in increased reserve mobilization, which leads to a hypothesis that metabolic signals decide the fate of starch breakdown. Starch mobilization was accelerated in a tuber when the bacterial sucrose isomerase gene was expressed in order to deplete sucrose content. Based on this result, the authors hypothesized that low sucrose levels trigger starch mobilization in stored potato tubers.

19.4.2 Phosphofructokinase

One of the mechanisms suggested for cold-induced accumulation of sugars in plants is cold sensitivity of phosphofructokinase. Phosphofructokinase converts fructose-6-phosphate to fructose-1,6-biphosphate, which is the first committed step in glycolysis. As a result, hexose-P sugars accumulate at low temperature and get diverted into the sucrose synthesis pathway (Trevanion and Kruger, 1991). Hammond et al. (1990) concurred with this hypothesis

by showing phosphofructokinase from two potato cultivars differing in their temperature responses and their difference in susceptibility to CIS. Consequently, at low temperatures, glycolysis is restricted and thereby decreases the amount of glucose that enters the respiratory pathway. Burrell et al. (1994) concluded that respiration in potato is not limited by phosphofructokinase. This was based on results obtained from transgenic tubers expressing *Escherichia coli pfk*A gene with a patatin promoter. The theory of cold-labile enzymatic steps in glycolysis favors the reducing sugar accumulation, when tubers were kept under 4°C did not find enough evidence (Hill et al., 1996). Malone et al. (2006) concluded that cold lability of glycolytic enzyme phosphofructokinase is not a major factor in sugar accumulation at cold temperature. The authors hypothesized that repression of glycolysis and carbohydrate oxidation has no role in the accumulation of hexose phosphates because there is no preferential inhibition of recycling of triose and hexose phosphates at low temperature.

19.4.3 Sucrose phosphate synthase

Another possible way of accumulating reducing sugar is by increased activity of sucrose synthetic enzymes. SPS is involved in synthesis of sucrose-6-phosphate from UDP-glucose. This reaction is followed immediately by dephosphorylation to form free sucrose. Reimholz et al. (1997) showed a novel form of SPS enzyme involved in the CIS process. The time course and temperature dependence of the appearance of this novel form of SPS correlates with sugar accumulation (Deiting et al., 1998). Krause et al. (1998) studied the SPS role using antisense and cosuppression techniques, and concluded that increased sucrose production at low temperature is because of changes in the kinetic properties of SPS, rather than an increase in the catalytic capacity. A 70–80% reduction in the SPS expression using the above-mentioned methods resulted in only a 10–40% decrease of soluble sugars in tubers stored at cold temperature.

By analyzing isozymes of targeted genes in greater detail, specific isoforms of SPS and a novel isoform of β-amylase (debranching enzyme) have been identified (Hill et al., 1996; Nielsen et al., 1997). This novel isoform β-amylase was isolated from the cultivar Desiŕee induced in tubers when transferred to cold (Nielsen et al., 1997). The time course and temperature dependence of induction of this β-amylase in potato tuber is similar to CIS (Deiting et al., 1998).

19.4.4 ADP-glucose pyrophosphorylase

The synthesis of starch in plant cells begins with the enzyme ADP-glucose pyrophosphorylase (AGPase), which catalyzes the reaction of glucose-1-phosphate with ATP to form ADP-glucose (liberating pyrophosphate) (Fu et al., 1998). The ADP-glucose is a substrate for starch synthase enzymes, which add glucose units to the end of a growing polymer chain to build up a starch molecule (releasing the ADP). Using the antisense approach, the importance of the AGPase role in starch biosynthesis is understood (Knutzon et al., 1992). Overexpressing AGPase resulted in an increase in starch content in tubers (Stark et al., 1992; Ballicora et al., 1995). An increase in the rate of starch biosynthesis resulted in increased the capacity of the tubers to degrade starch as measured by [U-14C] sucrose. It

is believed that the activity of AGPase in the starch synthesis is inhibited by this feedback system (Sweetlove et al., 1999).

19.4.5 Uridine-5-diphosphoglucose pyrophorylase

Uridine-5-diphosphoglucose pyrophorylase (UGPase) catalyzes the first step in the formation of sucrose. Hill et al. (1996) showed the correlation between the amount of UDP-glucose and sucrose levels during the process of CIS. Antisense constructs using UGPase showed a decrease in sucrose levels in potato tubers during cold storage (Spychalla et al., 1994; Borovkov et al., 1996). This is a committed step in the CIS process, and UDP-glucose limits the formation of sucrose in tubers (Sowokinos et al., 1997, 2000). By analyzing the number of susceptible and resistant CIS clones, Sowokinos et al. (1997) found allelic polymorphism at the UGPase locus. Two cDNA clones for UGPase differing in the BamHI site have been cloned (Spychalla et al., 1994). The UGPase allele UgpB showed certain isozyme of UGPase involvement in CIS process. Also, small differences in the cDNA sequence of UGPases in potato have previously been identified and explained as resulting from allelic polymorphism(Sowokinos et al., 1997).

Sowokinos (2001) suggested that cultivars that are less prone to CIS might have a higher rate of respiration in cold storage compared to those that are prone to CIS. Barichell et al. (1991) showed that the cold-resistant potato clone ND860-2 has a higher respiratory rate when compared to the cold-susceptible cultivar Norchip in storage. When tubers were subjected to low temperatures, the respiration rate in the tubers declines after the initial burst (Isherwood, 1973). Sherman and Ewing (1983) attributed this initial respiratory burst to cytochrome mediated and alternative oxidase-mediated pathways. At cold temperatures, the cell walls lose their fluid character, which results in leakage from cellular membranes. The plant uncoupling mitochondrial protein (PUMP) is strongly induced when tubers are exposed to cold temperatures. The PUMP is also believed to reduce oxidative stress at low temperatures (Nantes et al., 1999). Gounaris and Sowokinos (1992) isolated and tested mitochondria from tubers that were resistant and susceptible to CIS. Tubers that accumulated higher amounts of reducing sugar tend to have mitochondria with lower-buoyant density when compared with resistant cultivars. It is speculated that this phenomenon is due to alterations in the permeability of the inner mitochondrial membrane.

CIS in tubers can be reversed by reconditioning tubers at elevated storage temperatures (Pritchard and Adam, 1994; Edwards et al., 2002). During the reconditioning period, the reducing sugar (glucose and fructose) concentration decreases in tubers and 80% of the reducing sugars are converted back to starch. The remaining 20% are lost through respiration. Response to reconditioning is a cultivar-dependent process, and some cultivars respond better than others. The other option suggested by Pritchard and Adam (1992) is the preconditioning of tubers at 15°C to limit the increase in reducing sugars during subsequent storage. Storage at temperatures of 20°C or above also leads to an increase in sugar levels due to increase in respiration and other biological processes (Linnemann et al., 1985). Conditions such as elevated temperature or low temperature can cause sugar accumulation in tubers. The sugar content rose slightly at 16°C, whereas reduction in sugar content was observed at 7°C. Potato tubers need to be stored at an ideal temperature range, specific for individual cultivars where equilibrium is achieved and the net production of free sugars is at its minimum (Kumar et al., 2004).

19.5 Sprouting

Regulating dormancy and sprouting is an important aspect of potato crop management because the tuber also represents the starting material for the next generation of plants (seed tubers). Potato seed storage management is different from the regular storage. Seed tubers can be stored at low temperatures unlike tubers meant for processing. Sprouting is one of the main causes for loss in stored potatoes. Sprouted tubers are not marketable and tend to lose water by evaporation, which results in loss of weight.

Potato-processing plants require high-quality tubers for year-round operations. To ensure constant supply from storage, tuber dormancy needs to be extended beyond the winter months. During an extended storage period, tubers age physiologically, break dormancy, and sprout. Major factors for deterioration in the processing quality of tubers in storage are weight loss due to respiration; water and turgor loss due to sprouting; and an increase in reducing sugar concentration due to starch conversion.

19.5.1 Mechanism of sprouting

The suppression of bud growth in dormant potato tubers is believed to be under hormonal regulation (Suttle, 1996). The major plant hormones playing an active roles in determining bud dormancy are gibberellins (Suttle, 1996), cytokinins (Suttle and Banowetz, 2000), abscisic acid (Suttle, 1995), and indoleacetic acid (Sorce et al., 2000). Before the onset of dormancy, ABA levels are higher and gradually decrease as dormancy progresses. It is believed that ABA levels act as a potential signal for breaking dormancy. It has been proposed that initiation of sprouting occurs when ABA levels fall below a threshold value. So far no such threshold value for ABA to maintain tuber dormancy has been found (Destefano-Beltrán et al., 2006a, b). In contrast to earlier reports, Sorce et al. (1996) found that ABA concentration in sprouting eyes goes up during the release from dormancy. Destefano-Beltrán et al. (2006a) analyzed the expression pattern of both ABA biosynthetic and degradative pathway genes during the process of dormancy and sprouting. Destefano-Beltrán et al. (2006a) hypothesized that ABA levels are maintained in a tuber with respect to its physiological age by activation of biosynthetic and catabolic pathways. Claassens et al. (2005) showed that ethanol and other primary alcohols can break dormancy of apical bud tissue, and this action can be inhibited by alcohol dehydrogenase inhibitor. ABA could reverse the effect of ethanol, suggesting that ethanol may lower endogenous ABA levels, by promoting sprouting.

Recently, there are a few reports in the literature comparing gene expression during sprouting and tuber formation (Claassens, 2002; Verhees, 2002; Ronning et al., 2003). Ronning et al. (2003) compared potato-EST libraries from various tuber developmental stages. Some of these genes that are upregulated during the sprouting process are involved in offering protection against oxidative stress in plants such as putative glutathione transferase and glutathione-dependent dehydroascorbate reductase. These enzymes are involved in glutathione metabolism and turnover. However, Rojas-Beltran et al. (2000) found relatively little change in expression of antioxidant genes during sprouting. Upregulation of two starch biosynthetic genes (AGPase and granule-bound starch synthase (GBSS)) during sprouting was found (Claassens; Verhees, 2002; Ronning et al., 2003). Verhees et al. (2002) showed that expression of cell cycle–related genes and AGPase increased specifically in the

eye using a luciferase reporter gene. Similarly, lipoxygenase expression increased both during sprouting and tuberization (Ronning et al., 2003). Interestingly, when transgenic plants expressing inorganic pyrophosphatase gene with tuber-specific promoter, tubers sprouted 6–7 weeks earlier than control plants (Farré et al., 2001). Authors hypothesized that increased mobilization of starch to sucrose led to an accelerated sprouting phenotype. However, Hajirezaei and Sonnewald (1999) reported that tubers from transgenic plants overexpressing pyrophosphatase never sprouted. The reason suggested by the authors was a complete shutdown of glycolysis due to the inhibition of pyrophosphate-dependent phosphofructokinase.

During the sprouting process, the mother tuber supplies energy to the growing sprout by mobilization of starch reserves. Enzymes that are involved in starch mobilization are upregulated. At the time of sprouting, amylase enzyme activity involved in starch break down increases near the tuber eye tissue (Bailey et al., 1978; Biemelt et al., 2000). However, Davies and Viola, 1988 reported a decrease in total amylase activity around the time of sprouting. Thus, Biemelt et al. (2000) concluded that there is no clear-cut evidence for an increase in starch-degrading enzymes around the time of tuber sprouting. Similar gene expression patterns are also observed in the case of starch synthesis enzymes during the sprouting process, contrary to the expectations (Claassens; Verhees, 2002; Ronning et al., 2003). The starch biosynthetic enzyme (AGPase) and starch-degrading enzyme (amylase) are active during both the sprouting and tuberization processes (Vreugdenhil, 2004). Vreugdenhil (2004) suggested that the biochemical machinery for starch synthesis and breakdown is present during all stages of development of the tuber, including sprouting, and is coordinately up- or downregulated based on flux and metabolite concentration.

19.5.2 Physiological aging influences sprouting

Potatoes have a set period of dormancy before they sprout depending on the cultivar. The precise timing for sprout initiation however depends on the age of the tuber. The physiological age is different from chronological age. Physiological age has a greater impact on sprouting and the number of stems per tuber, and it is the primary factor that determines viability of tubers used for seed. Physiological age is a cumulative effect of biochemical changes taking place within a tuber (Bohl et al., 1995). Factors that influence physiological aging are growing conditions, storage conditions, and wounding. Growing conditions such as low moisture, high temperatures, fertilizer, frost damage, and disease pressure may all cause stress on the potato. Wounding and bruising during harvest and the cutting of seed tubers before planting increases the respiration rate, which results the tuber stress and causes aging of the tuber. Respiration levels remain low at low-temperature storages. Any fluctuations from an ideal storage temperature may also rapidly age the tubers. However, the major aging of seed tubers occurs during storage.

The physiological age of seed can be determined by leaving the tubers at room temperature in dark conditions and by assaying the number of days required to sprout and number of sprouts per tuber (van der Zaag and van Loon, 1987). There is no biochemical marker available to determine physiological age. Knowles et al. (2003) suggested that 2-methyl butanol can be used as a marker but needs to be tested further for its potential use. Heat accumulation model measures aging by counting number of degree days or the number of the heat units tubers might have exposed (Knowles and Botar, 1991; Jenkins et al., 1993).

Accumulated heat units, also known as day-degrees and degree-days, are calculated by taking the average daily temperature from each day and subtracting the growing base temperature (8°C) (Allen and Brien, 1986). The heat units for each day are then added over time to provide accumulated heat units. This requires information on growing and harvest conditions, handling procedures, and the storage environment (Mac Kerron, 1998).

The physiological age of the potato seed influences performance of the crop (Reust, 1986). Seed produced at low temperature, moisture or fertility stress, disease pressure, and stored under fluctuating temperatures could result in physiologically old seed, which is beneficial under short growing season conditions in terms of vigor. Environmental conditions need to be considered when determining how to manage physiologically young or old seed (vander Zaag and van Loon, 1987). Physiologically old seed usually sprouts quicker than young seed. The emergence rate under cool weather conditions is quicker for physiologically old seed compared to young seed. So if both types of seed are stored at the same temperature, emergence of younger seed is slower. Early emergence of plants is desirable when potential problems such as seed piece decay, *Rhizoctonia* stem canker, and other soil-based pathogen infection (Bohl et al., 1995). Seed potato producers typically try for higher yield with a smaller tuber profile, whereas commercial growers have different tuber size and total yield expectations. Physiologically old seed with early emergence of sprouts and higher sprout numbers is a better option for seed growers (Asiedu et al., 2003). The other important aspect concerning physiologically old seed relates to seed spacing. In order to decrease the number of stems per acre and to maintain plant vigor, physiologically old seed requires increase seed spacing at planting (Kleinkopf and Barta, 1991).

19.5.3 Sprout-stimulating agents

Potatoes sprout as a result of stimulation from the environment such as light and temperature. Often seed potatoes are treated with chemicals to hasten the dormancy break for sprout growth. This is needed in regions where two potato crops are grown each year, when seed tubers are exported to different climatic regions for planting, and when it is advantageous to reduce dormancy period for planting. The chemicals commonly used for dormancy break are ethylene, carbon disulfide (CS2), rindite, gibberellic acid (GA3), and thiourea. Sucrose content is higher in tubers treated with GA3, CS2, and rindite as compared to thiourea (Rehman et al., 2003). Breaking dormancy in thiourea-treated tubers is slower due to delay in starch mobilization compared to other treatments

GA3 is applied by spraying or dipping tubers in a solution. GA3 is applied immediately after harvest or after seed cutting for better absorption through the wounded areas. This treatment increases the number of stems resulting in smaller tuber size (Kustiati et al., 2005). Rindite is applied as a vapor prepared by mixing ethylene chlorohydrin, ethylene dichloride, and carbon tetrachloride in 7:3:1 ratio. Treatment should be in a closed container because this is also toxic substance. Tubers are exposed to vapors for 48 h. Decaying has been reported when treated with rindite without wound healing for 14 days (Kim et al., 1999). Rehman et al. (2003) compared different treatments for breaking dormancy in microtubers and found that rindite treatment resulted in the highest sprout ratio in all cultivars tested and tubers sprouted earlier (10 days) compared to other treatments. Decay has been reported in tubers treated with thiourea (Rehman et al., 2003). Carbon disulfide was also tested for the same, but its usage is minimal because but it is toxic.

19.5.4 Potato sprout inhibitors

Physiological dormancy of the potato tuber is further extended with an artificial environment consisting of lowered temperature, high relative humidity, adequate oxygen, and by application of chemical sprout inhibitors. The purpose of sprout inhibitors is to prevent sprouting in storage as tubers age. Sprout inhibitors work by inhibiting cell division within the eye region; therefore, they should never be applied to seed potatoes. Ideally, seed storage should be in separate units with independent air systems; if this is not possible, then care needs to be taken to avoid drift or movement of sprout inhibitors into areas where seed potatoes are present. Sprouting causes reduction in the quality of the tuber. This leads to weight loss due to water loss and disease due to reduced air circulation (Afek et al., 2000).

The more widely used sprout inhibitors are maleic hydrazide and chlorpropham (isopropyl *N*-(3-chlorophenyl)carbamate) (CIPC). Substituted naphthalenes are also used as short-term sprout suppressants on seed. Due to recent Environmental Protection Agency (EPA) standards and more awareness regarding these chemical suppressants, alternatives have been explored. Natural compounds, such as carvone and clove oil, have shown effectiveness in sprout suppression. Ethylene is also gaining popularity as a sprout suppressant in Europe and Canada for obvious health reasons, but it is also associated with darkening in french fry color.

19.5.5 CIPC

CIPC is applied as an aerosol by a trained technician for treating potatoes in storage. CIPC may also be applied as an emulsifiable concentrate on potatoes coming out of storage as they are packed. It is a potent inhibitor of cell division specifically interrupting spindle formation during mitosis (Vaughn and Lehnen, 1991). It is advisable to enter treated bins only after 10 complete air exchanges. CIPC application is common after natural tuber dormancy ends and before sprouting starts. This is typically 75–120 days after harvest depending on cultivar. CIPC can be effective up to a year after treatment. This irreversible sprout inhibitor is mostly used on tubers intended for fresh market and the processing industry. Seed potatoes should not be stored in the same room where CIPC was recently used. CIPC-treated storage rooms should be cleared thoroughly including the air systems and ducts before storing seed potatoes. Treated tubers should be held at least 30 days in storage before marketing. The allowable residue levels of CIPC on tubers is 30 ppm per fresh weight in the United States (EPA), and this product has been successful for more than 40 years as a sprout inhibitor (Kleinkopf et al., 2003). In Europe, Australia, and Canada, maximum allowable limit for CIPC residue is 5–10 ppm per fresh weight. This requires multiple applications for long-term storage (Kleinkopf et al., 2003). Effective sprout inhibition with CIPC can be achieved at 1–2 ppm concentration (Kleinkopf et al., 1997). Single CIPC application under ideal storage conditions maintains tubers for 4–5 months, while a second application is needed to extend sprout inhibition beyond 8 months (Kleinkopf et al., 2003). The sprout inhibition response to CIPC application depends on storage temperature, cultivar, and other stress factors during the growing season (Brandt et al., 2003). Generally, at lower temperatures, tubers store longer with CIPC application. Even with processing cultivars such as Russet Burbank that are stored at 10°C can maintain quality into late April (Kleinkopf et al., 2003).

Lentaza-Rizos and Balokas (2001) measured the residue concentration of potatoes treated with CIPC. Residue levels in tubers decrease by 20% after 1 month of storage, and 40% after 65 days of storage. Their studies showed washing in water for minute removes 88% and peeling removes 91–98% of total residue (Tsumura-Hasegawa et al., 1992; Lentaza-Rizos and Balokas, 2001). Internal sprouting is reported occasionally in commercial lots treated with CIPC. This is attributed to the unequal distribution of CIPC in the commercial storage.

19.5.6 Maleic hydrazide

Maleic hydrazide is applied as foliar chemical in potato, onion, and tobacco to inhibit sprouting (Gichohi and Pritchard, 1995). Maleic hydrazide is applied to the growing potato crop, and is translocated to the developing tubers where it arrests cell division, but does not limit cell expansion. Early application during tuber development can be problematic, and will limit tuber size and yield. Best results were obtained when the chemical was applied around 2 weeks before vine killing. Ideal daytime temperatures at the time of application is less than 85°F. Irrigation or rainfall within 24 h of application may reduce effectiveness (Weiss et al., 1980). Caldiz (2001) showed sprout inhibition with maleic hydrazide was possible for 8 months with no loss of weight and tubers could produce good-quality french fries. Maleic hydrazide delays the initial sprouting date, but there is no reduction in yield when used on seed potatoes (Caldiz, 2001). Disorders associated with maleic hydrazide are bud end cracking and elephant hide on tubers due to uneven application. Maleic hydrazide is applied only once in a season, and plants should not be under stress conditions when it is applied.

19.5.7 1,4-DMN

Ethyl substituted naphthalenes are naturally occurring in potato tubers and contribute to flavor in baked potatoes (Buttery et al., 1970; Coleman et al., 1981). These compounds showed sprout-suppressant activity on a short-term basis approximately for 30 days. Different isomers of dimethylnaphthalene (DMN) and diisopropylnaphthalene (DIPN) were tested and compared for their sprout-suppressant activity by Lewis et al. (1997). Lewis et al. (1997) found that DIPN was more efficient than DMN in sprout suppression. Mode of action of these compounds for sprout suppression is by regulating phytohormones (Kleinkopf et al., 2003). The short-term nature of the sprout-suppressant activity of 1,4-DMN allows for a potential use in the seed industry. Diisopropylnaphthalene is also applied as an aerosol for treating potatoes in storage. The active ingredient in 1,4-Sight™ and 1,4-Ship™ is 1,4-dimethylnaphthalene, and Amplify™ is 2,6-diisopropylnaphthalene. 1,4-DMN is marketed specifically to control sprouting of seed during storage and transit. Beveridge et al. (1981a) was the first to suggest that 1,4-DMN has an effect on tuber size distribution in a crop growing from treated seed potatoes and showed its concentration dependence. DMN is applied after the wound healing (curing) process and natural tuber dormancy ends and before sprouting occurs. This product is custom applied and highly toxic to fish. Care must be taken in disposing of water that is used in the storage bins treated with DMN.

The different mode of action of DMN is exploited by the potato storage industry by alternating with the popular sprout suppressant, CIPC, for long-term storage needs. The

synergistic mode of action between these two compounds for sprout suppression was reported by Riggle and Schafer (1997) and Beaver et al. (2003). This approach led to reducing the required concentration of CIPC to achieve effective sprout suppression in potato storages. The study conducted by Beaver et al. (2003) showed a 50% reduction in CIPC levels when applied at 1:1 ratio with DIPN with the same sprout-suppressant activity.

Multiple application of 1,4-DMN delayed the emergence in most of the cultivars studied and also reduced tuber size and increased stem number. The effect and extent of increase in tuber number and reduction in size depends on the cultivar (Knowles et al., 2005). Nolte conducted a similar study with 1,4-DMN using a single application (P. Nolte, personal communication). His results also showed delay in plant emergence, but did not find any change in tuber size distribution.

19.5.8 Irradiation

High-energy irradiation to inhibit potato sprouts is in limited usage in a few countries like Japan, the Netherlands, and Canada (Thomas, 1984). The widespread use of this technology has been impeded by consumer acceptance to irradiated produce. Burton and Hannan (1957) reported 50–100 Gy or even lower doses were highly effective in total sprout inhibition. Exposing potato tubers to high-energy radiation causes an increase in starch solubility, decrease in starch swelling power, and viscosity (Farkas et al., 1987, 1988). These changes in starch are attributed to depolymerization of starch and modification of amylose and amylopectin structure (Duparte and Rupnow, 1994). Al-Kahtani et al. (2000) conducted a similar study using a Co60 gamma ray semicommercial irradiator at a dose rate of 900 rads/min (0.05–0.20 kGy) on potato tubers. The study revealed changes in starch characteristics, which are dependent on irradiation dose, timing, cultivar, and postirradiation conditions. Frazer et al. (2006) reported successful sprout suppression with 40–50 Gy dosage using an 18-MeV industrial-type linear accelerator on Russet Burbank. Sprout suppression was observed for 6–8 months in storage at 7.2°C. Immediately after irradiation, glucose levels were higher, but storing at a slightly high temperature for 2–6 months reduced the effect. Tubers recovered quickly when irradiated with a higher dosage for a shorter time compared a lower dosage for a longer time. Tubers treated with the higher dosage (100 Gy) showed higher soft rot and dry rot incidence. This is attributed to the inhibition of the wound periderm process (Thomas, 1982). Tuber lots with high disease susceptibility may not be suitable for irradiation, and freshly harvested tubers tend to do better with irradiation treatment.

19.5.9 Alternative sprout inhibitors

Essential oils, monoterpenes, and other volatile organic compounds extracted from plants were tested to find alternative and more environment-friendly sprout inhibitors for their effectiveness on sprout inhibition (Oosterhaven et al., 1995b; Sorce et al., 1997). These alternative sprout suppressants are most effective when they are applied at “peeping,” or before sprouts are one-eighth of inch long (Kleinkopf et al., 2003). These materials need to be applied multiple times during storage to maintain tuber dormancy. Application timing is critical for the success of sprout inhibition. Added advantages with these materials are that they also suppress disease in storage (Farag, 1989; Thompson, 1989; Vokou,

1993). *S*-(+)-carvone, a monoterpene extracted from caraway oil, is the first commercially marketed (Talent^{TM}) plant-based product for sprout suppression in Europe. Continuous treatment inhibits tuber sprouting (Oosterhaven et al., 1995a, b). Jasmonates and its derivative compounds showed potential postharvest applications in fruits and vegetables (Buta and Moline, 1998; Droby et al., 1999). The response to jasmonates in sprout suppression activity of potato tubers is ambiguous (Lulai et al., 1995; Oberg and Klienkopf, 2000).

Coleman et al. (2001) examined ethnobotanical and natural products literature on aromatic herbs in the northern Andean regions of South America, for food preservation, insect repellant, and sprout inhibition properties. Based on the literature, they compared the active ingredients in those plant species, *S*-(+)-carvone, with menthone and neomenthol for sprout inhibition in potatoes. Menthone and neomenthone are 5–10 times more effective than *S*-(+)-carvone in sprout suppression when applied in combination at a 0.5 ppm concentration level in laboratory-scale experiments for 4 weeks at 10°C. Method of application is by direct vapor, but the authors suggested using a porous polysterene matrix due to ease of handling and application. Tubers maintained all acceptable processing quality characteristics with these treatments.

Eugenol is an extract from clove (*Syzygium aromaticum* L.) marketed as Biox-A^{TM}. This is another plant-derived compound that is commercially marketed for sprout inhibition (Kleinkopf and Frazier, 2002). It has an organic label from many states in the United States. It is applied as thermal aerosol, and multiple applications are required. The recommended dosage for a 60-day storage period is 90 ppm followed by 30 ppm after 3-week gap (Kleinkopf et al., 2003). Sprout suppression is by physical and chemical damage to the developing sprouts and buds.

Frazier et al. (1998, 2000) tested mint oils (spearmint and peppermint) for sprout inhibition in potato storage. Mint oils need to be applied continuously for effective sprout suppression. Cold aerosol and forced evaporation are more effective treatments when compared to thermal fogging (Kleinkopf and Frazier, 2002).

For successful commercial application of these naturally occurring volatile compounds as alternative sprout inhibitors, there is a need to test these compounds for their consumer acceptability because they possess distinct aroma and flavor characteristics. Boylston et al. (2001) tested the sensory quality of potatoes treated with different sprout inhibitors such as salicylaldehyde, 1,8-cineole, and 1,4-dimethylnaphthalene and compared these with CIPC-treated tubers. The evaluation was carried out using a sensory panel during a 16-week storage period. This study revealed that tubers treated with 1,8-cineole and salicylaldehyde can be differentiated from untreated or CIPC-treated tubers. No difference in sensory perception was detected with 1,4-dimethylnaphthalene-treated tubers.

19.5.10 Ethylene

In the literature, conflicting results were published on the role of exogenous ethylene in potato tuber dormancy. Depending on the concentration and duration, ethylene can either extend or shorten dormancy. Short-term treatment of exogenous ethylene treatments reduced dormancy length, and continuous treatment resulted in suppression of subsequent sprout growth (Timm, 1986). The first long-term application of ethylene for sprout inhibition was reported by Metlitskii et al. (1982). Prange et al. (1998) first published a 3-year study on commercial application of ethylene using the Russet Burbank cultivar. By continuous

treatment at 4 μL/L (166 μmol/m^3) concentration, sprouting was delayed in tubers for 25 weeks. Ethylene treatment results in uniform sprouting from all eyes when the tubers are reconditioned at room temperature. Ethylene has been registered as "Eco Sprout Guard^{TM}" as a potato tuber sprout-suppressant in Canada (Daniels-Lake et al., 2005).

Prange et al. (1998) hypothesized that continuous treatment of ethylene terminates bud rest at biochemical and cellular levels but stops further cellular differentiation and elongation. Sprouts that are formed after treating with ethylene are easy to detach from tuber compared to untreated tubers. Ethylene exposure increases polyamine levels in tubers (Jeong, 2002). Increased levels of spermidine in stored tubers produce more smaller-size tubers (Pedros et al., 1999). Daniels-Lake et al. (2005) tested different concentrations of ethylene on fry color. All the ethylene treatments in storage darkened the fry color compared to CIPC treatment (Prange et al., 2001; Daniels-Lake et al., 2005). This darkening is due to increase in polyamine levels in tubers. Application of 1-methylcyclopropene (1-MCP) as a pretreatment before applying ethylene improved fry color (Prange et al., 2001, 2005). 1-MCP is applied at 1 μL/L for 2 days prior to ethylene treatment. Some cultivars (Shepody) require multiple treatments of 1-MCP during storage to reduce fry color darkening.

Endogenous ethylene plays an important role in microtuber endodormancy (Suttle, 1998a, b). Dose-dependent treatments using an ethylene noncompetitive antagonist such as silver nitrate ($AgNO_3$) and a competitive antagonist such as 2,5-norbornadiene (NBD) initiated sprouting in potato tubers. Premature tuberization was 98% when treated with $AgNO_3$ at the 50 μM concentration, and 85% when treated with NBD at 5 ppm concentration. Ethylene inhibitors are effective in inducing sprouting if tubers are treated early. This led to the hypothesis that ethylene is required only to initiate endodormancy in microtubers. Ethylene can reverse these changes, which further confirms the role of ethylene in promoting dormancy.

Hydrogen peroxide–based materials suppress the sprouting by causing physical damage to the sprout tips and buds (Afek et al., 2000). These materials are applied with an atomizing system after wound healing in potatoes. Afek et al. (2000) compared sprout inhibition activity of CIPC with hydrogen peroxide. Four times treatment with 10% hydrogen peroxide resulted in 0% sprouting even after 6 months of storage (Afek et al., 2000). López-Delgado et al. (2005) showed spraying hydrogen peroxide in field increased starch concentration in tubers to 6–30%, and it will be highly interesting to see how these tubers perform in storage.

19.6 Conclusions

In summary, there are considerable advances made in understanding the biology of dormancy and sprouting in plant systems. Additionally, the practical benefit in understanding the biological process behind wound healing, CIS, and physiological aging will be enormous. The key is to understand how different plant hormones interact together to orchestrate these biological phenomena. Gaining a complete understanding of the genetics of such complex traits is central to our ability to use biotechnology for the improvement of potato. More information on specific plant hormone roles and their cross talk in these processes will help in developing new environmental-friendly technologies to manipulate both dormancy and sprouting. Using multiparallel technologies such as genomics, proteomics, and metabolomics can give us new insights to manipulate metabolites.

Significant advances have been made in using potato for nonfood applications. Genetic and molecular approaches are used to manipulate carbohydrate metabolism in tuber, thereby changing tuber starch content and amylopectin levels (Regierer et al., 2002). Using potato for therapeutic molecule production and edible, plant-based vaccination system and biodegradable plastics production will offer new and exciting challenges to potato postharvest biology (Kim et al., 2004; Neumann et al., 2005; Twyman et al., 2005).

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Chapter 20

Biosensor-Based Technologies for the Evaluation of Quality

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Abbreviations used

Ala, alanine; BSA, bovine serum albumin; CDR, complementarity-determining region; CFU, colony-forming unit; ELISA, enzyme-linked immunosorbent assay; EU, European Union; HRP, horseradish peroxidase; Met, methionine; MRL, maximum residue limit; NTA, nitrilotriacetic acid; Pro, proline; RI, refractive index; RU, response units; SIRE, sensors based on injection of the recognition element; SPR, surface plasmon resonance; TIR, total internal reflection.

Structures

A list of chemical structures is provided in the Appendix. These are molecules that are referred to in the text and are relevant.

20.1 Introduction

The monitoring of quality of agricultural produce is of huge importance for farmers, the food industry, legislators, and, most importantly, for consumers (Karlsson, 2004). The quality of fruits and vegetables may be compromised by the presence of toxic materials such as herbicides and pesticides. The extensive use of such compounds to suppress the growth of plant and insect populations, respectively, implies that consumers could be exposed, on a daily basis, to a vast number of different toxic chemicals, if food produce is not carefully monitored. The presence of mycotoxins, such as aflatoxin B_1, also poses a potential threat to consumers. Over recent years, public concern has increased with regard to the possible health risks associated with long-term and low-level exposure to various food-borne chemicals that could ultimately result in chronic toxicity (Keay and McNeil, 1998). The conditions in which fruit crops are stored are often the most important factors in determining the efficiency and profitability of the harvest (Shmulevich et al., 2003) and can influence contamination levels significantly. Hence, the development of methods to detect traces of potentially harmful contaminants is crucial for monitoring the overall quality of fruit and vegetable products.

20.2 Methods of analyzing quality

There are several standard methods that are currently used to monitor the quality of fruit and vegetable produce. Basic methods of inspection include the following:

- Visual monitoring (color, gloss, firmness, shape, and size of the product, as well as noting the presence of defects).
- Analysis of soluble solid content.
- Monitoring of titratable acidity.

Most of these tests can be carried out immediately after harvesting on location at minimal cost (Mitchum et al., 1996). However, if these tests are not sufficient for providing confirmation to the consumer that a product satisfies acceptable regulations (mentioned later), then it is a common practice for samples to be removed and sent to a central laboratory for comprehensive in situ analysis (Giraudi and Baggiani, 1994). These methods of analysis include the following:

- High-performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS). These analytical methods are accurate and highly sensitive, but are also time-consuming and beyond the analytical capacities of smaller operators (such as "on-site" laboratories) as they require expensive instrumentation, lengthy sample treatment, and trained personnel to analyze the data (Bäumner and Schmid, 1998).
- The detection of indicator molecules that represent product freshness, including flavonoids (MacLean et al., 2006).
- Monitoring of bitterness (Dourtoglou et al., 2006).
- Assessing product firmness (the Magness–Taylor test).
- A nondestructive test, measuring an acoustic response from fruit with the signal being interrogated using a fast Fourier transform, to determine elasticity (Shmulevich et al., 2003).
- Near-infrared spectroscopy (in a nondestructive, accurate, and rapid protocol) for the detection of mycotoxigenic fungi and their toxic metabolites that are commonly found in fruits and vegetables (Berardo et al., 2005).
- The use of scanning electron microscopy. Schirra et al. (2005) developed a method to inspect the ultrastructural changes of a fruit's epicuticular layer when the dip treatment pesticide, fludioxonil, was used to control *Penicillium* spp. infestations postharvest.
- Biosensor-based platforms, such as Biacore™ and electrochemical sensors.

20.3 Biosensor technologies

Biosensors have been particularly successful for monitoring the presence of a variety of analytes in fruit and vegetable produce (including pesticide and herbicide residues) that may be present in minute quantities. A biosensor (Fig. 20.1) can be defined as an analytical device that incorporates an immobilized biological element (see Table 20.1), which interacts with an analyte of interest in "real time" (Scheller and Schubert, 1992). The three main components of a biosensor are as follows:

- A biological recognition component,
- A transducer, and
- A readout device (such as a computer).

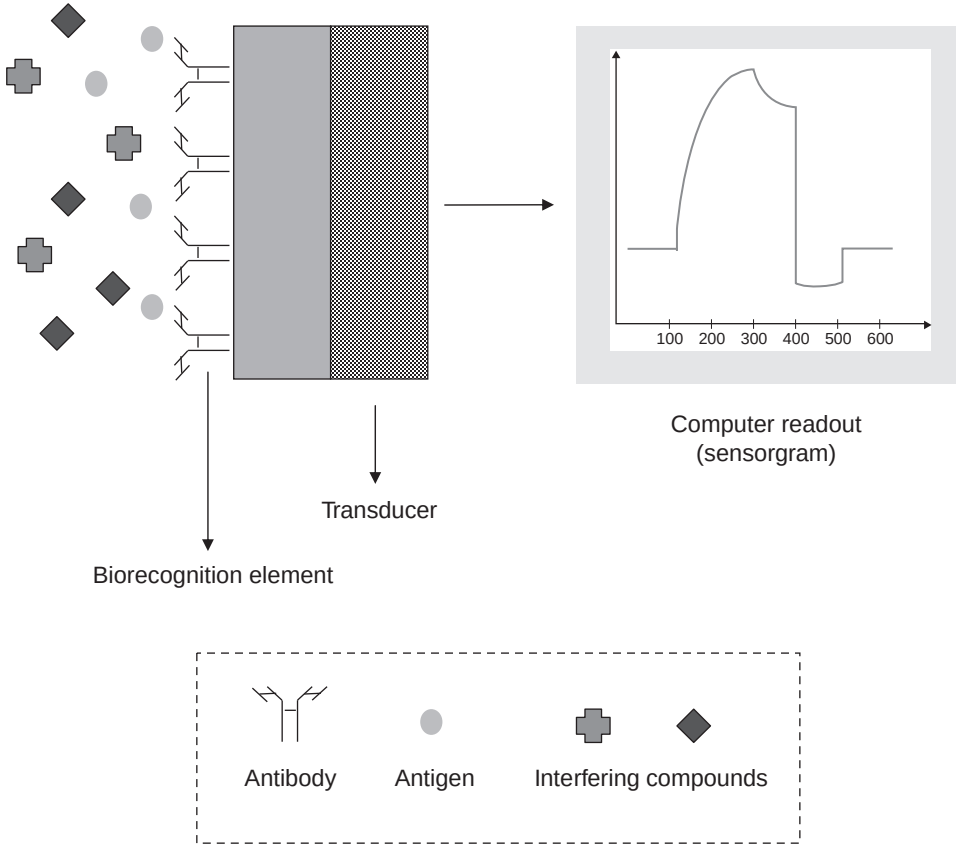


Fig. 20.1 General format of a biosensor. The biorecognition element is in spatial contact with the transducer, which converts the signal to an output shown on the computer.

A transducer (Table 20.2) is a device, such as a piezoelectric crystal, microphone, or photoelectric cell, which converts input energy of one form into output energy of another. The output signal generated is proportional to the concentration of the target analyte of interest (Luong et al., 1995). A readout device typically is composed of a computer-linked monitor that presents the data in the form of a sensorgram, which is a graph that illustrates

Table 20.1 The recognition elements commonly used in sensor systems

1.	Antibodies and antibody fragments—derived by enzyme digestion or genetic engineering (Fab fragment, scFv, and diabody)
2.	Enzymes—for example, those specific for one particular substrate (i.e., horseradish peroxidase for hydrogen peroxide)
3.	Cell membrane receptors
4.	A living cell—eukaryotic or prokaryotic
5.	Nucleic acid–based probes—DNA and RNA or peptide nucleic acid
6.	Aptamers
7.	Chemically generated recognition surfaces—including plastibodies (artificial antibodies or molecularly imprinted polymers)

Table 20.2 Examples of transducers commonly used in biosensor systems

Type	Example	Principle of use
Electrochemical	Conductimetric	Solutions containing ions conduct electricity. Depending on the reaction, the change in conductance is measured
	Potentiometric	Measurement of the potential of a cell when there is no current flowing to determine the concentration of an analyte
	Voltammetric	A changing potential is applied to a system, and the resulting change in current is measured
Field effect transistor-based	Field effect transistor	A current flows along a semiconductor from a source gate to a drain. A small change in gate voltage can cause a large variation in the current from the source to the drain
Optical	SPR	Surface plasmon resonance (a detailed explanation is given in the chapter)
Thermal	Calorimetry	Heat exchange is detected by thermistors and related to the rate of a reaction
Surface acoustic wave	Rayleigh surface wave	An immobilized sample on the surface of a crystal affects the transmission of a wave to a detector
Piezoelectric	Electrochemical quartz crystal microbalance	A vibrating crystal generates current that is affected by a material adsorbed onto its surface

the change in signal on an analytical chip surface over time resulting from the interaction between bound and mobile entities. A sample sensorgram for Biacore is illustrated in Fig. 20.2. The units of measurement used in the sensorgram are referred to as response or resonance units (RU). A response of 1,000 RU represents a change in the resonance angle of 0.1°, and is equal to a change in the surface coverage of the chip of $\sim$1 ng/mm^2.

The ideal characteristics of a biosensor are listed in Table 20.3. There is immense potential to broaden the scope and use of biosensors in many fields, such as in the monitoring of quality of postharvest fruit and vegetable produce. A review of relevant sensor systems, including the available commercial SPR instruments, was compiled by Leonard et al. (2003). There are many other SPR-based instruments in the market, such as the Spreeta system. This instrument is more cost-effective than other biosensors, but, as reported in the literature, it is less sensitive when used with real samples compared to larger instruments (Setford et al., 1999) such as Biacore. Biacore uses an optical-based transducer system for the measurement of analytes based on the principle of surface plasmon resonance (SPR). SPR works on the principle of total internal reflection (TIR), a phenomenon that occurs at the interface between two nonabsorbing materials such as water and a solid. When a source of light is directed at such an interface from a medium with a higher refractive index (RI) to a medium of lower RI (such as light traveling through glass and water), the light is refracted to the interface (Markey, 2000). When the light is above a particular angle of incidence, no light is refracted across the interface and TIR occurs. Even though the incident light is reflected back from the interface, an electromagnetic field (called an evanescent wave) penetrates a distance of the order of one wavelength traveling into the less optically dense medium. The Biacoresystem has a limit of detection of $\sim$10 RU (which is $\sim$10 pg/mm^2) and has an operating temperature range of 4–40°C for the Biacore 2000 model (Hashimoto, 2000). Additional instruments, such as the Biacore Q, have major potential for quality control

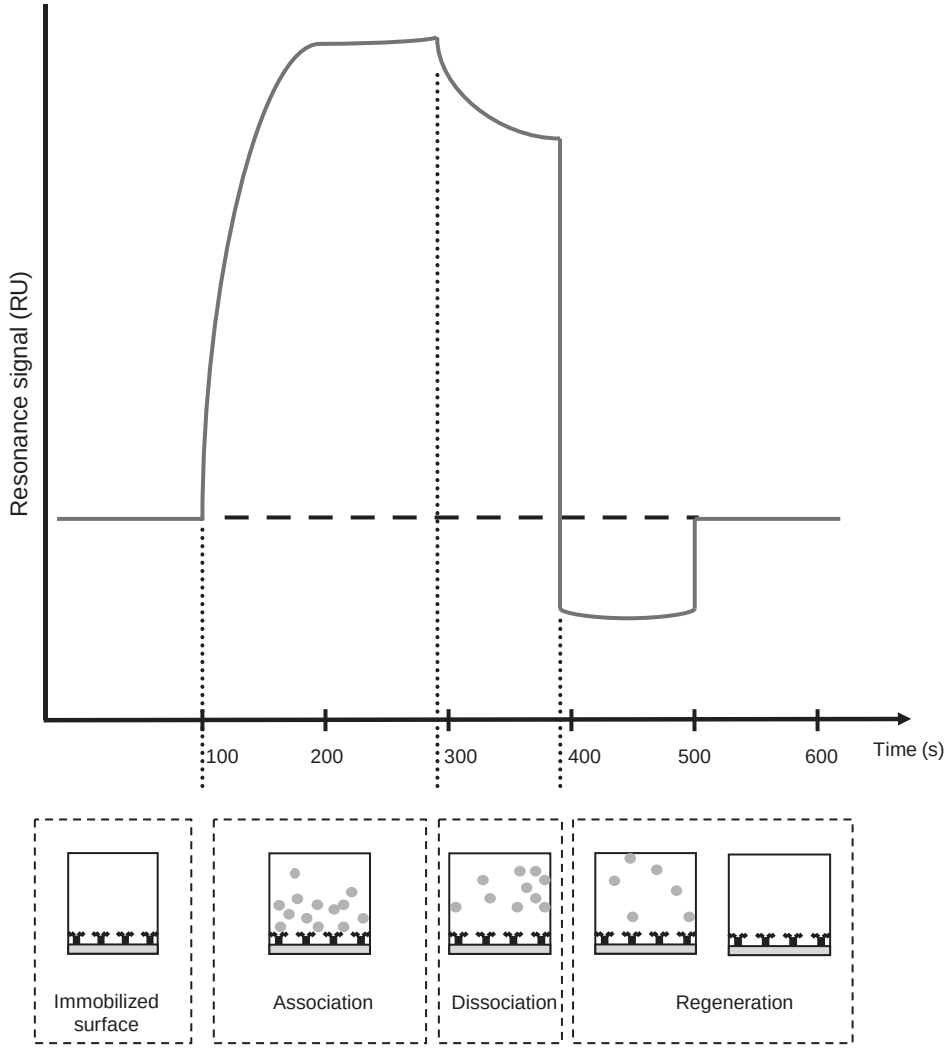


Fig. 20.2 Schematic representation of a Biacore sensorgram.

analysis, whereas the Biacore 3000 is particularly useful for high-sensitivity antibody-based analyte detection and kinetic analysis. The T100, and recently developed A100, permit high-throughput analysis of protein–protein and protein–ligand interactions, which may be useful in the judicious selection of antibodies for specific diagnostic applications. Systems such as Bio-Rad's ProteOn XPR36, Nomadic's SensíQ® formats and AlphaSniffer's common path interferometry SPR biosensor can also be applied for food quality control, though such applications are still in development.

An advantage of SPR over other optical methods of detection is that SPR measures the interaction between immobilized molecules on a surface (e.g., an antigen or an antibody immobilized onto a sensor chip) and the corresponding ligand in solution passing over this matrix. This means that the reaction can be measured in a colored solution or in a turbid

Table 20.3 The ideal characteristics of a biosensor

1.	The biorecognition element can be highly specific for the substrate or antigen
2.	Reusable, with multiple readings permitted on a single device
3.	High sensitivity
4.	Cost-effective
5.	Rapid analysis time
6.	Use of "on-line" or in situ measurement
7.	Results should be in "real-time"
8.	Good signal to noise ratio
9.	The device should be robust
10.	The ability to measure samples in a high-throughput fashion if required
11.	Possibility of incorporation into automated robotic systems
12.	Fast turnaround time on analysis
13.	Ease of use; ability of use in field by untrained personnel to provide a measurement

complex matrix (such as fruit or vegetable preparations). This is advantageous over other optical-measuring techniques where samples have to be specifically pretreated to remove contaminating colored species prior to analysis.

The correct choice of sensor chip is also an important factor to consider when designing an assay for any particular analyte. There are many different types of sensor chips that can be used with a Biacore instrument (Table 20.4). The choice of chip chemistry depends on many factors, including the application and objective of the test, the chemical characteristics of the biomolecules of interest, and their specific interactions:

- The CM5 chip is the most versatile biosensor chip currently available and is the most frequently used. Its matrix consists of a completely modified carboxymethylated dextran covalently attached to the gold surface (Fig. 20.3). Interactions involving small organic molecules, such as pesticides, large molecular complexes, or whole viruses, can be studied with this chip. The analytes of interest can be covalently coupled to the sensor surface through amine, thiol, aldehyde, or carboxyl groups. The chip has a high-binding capacity,

Table 20.4 The surface chemistries of available Biacore chips

Chip type	Modification type	Applications
CM5	100% carboxylation of dextran surface	General use
CM4	30% carboxylation of dextran surface	Serum, cell extracts
CM3	100% carboxylation of dextran surface	Serum, cell extracts
C1	100% carboxylation of dextran surface	Bacteriophage binding
L1	Lipophilic substances	Lipid capturing
SA	Streptavidin surface	Detection of biotin-containing molecules
NTA	Nickel–nitrilotriacetic acid	Detection of histidine-tagged molecules
HPA	Flat hydrophobic surface	Used for membrane-associated interactions
Au and SIA	None	Used in the study of surface chemistry interactions of self-assembled monolayers and interactions between surface materials and biomolecules

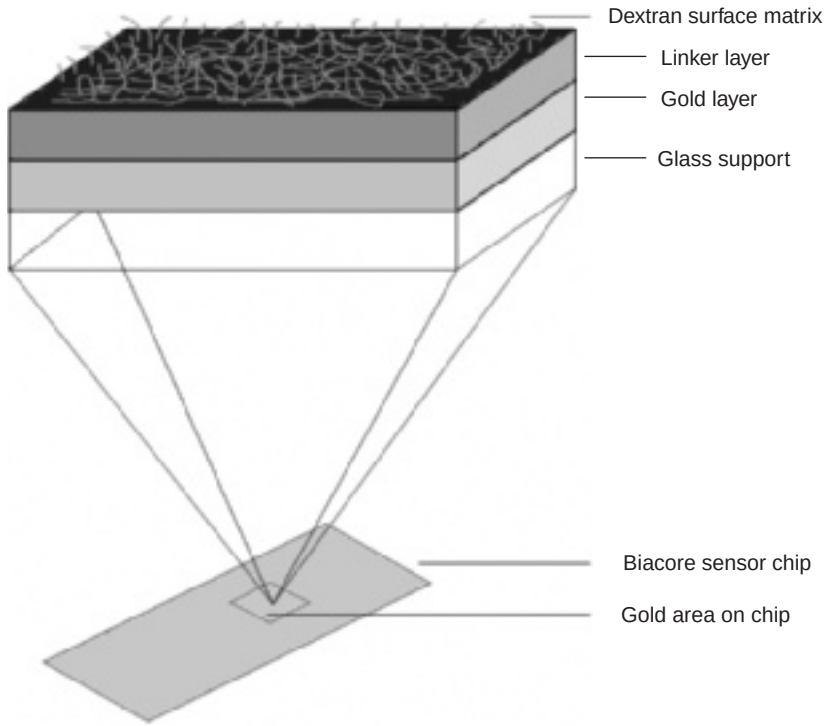


Fig. 20.3 A typical Biacore chip with the binding surface enlarged.

which results in a high response that is advantageous for capture assays, especially for those involving the interactions of small molecules (see later). The high-surface stability permits accuracy and precision, and repeated analysis on the same surface may be performed (Dillon et al., 2005).

- The streptavidin (SA) chip has a carboxymethylated dextran matrix preimmobilized with streptavidin for the immobilization of biotinylated peptides, proteins, nucleic acids, and carbohydrates.
- The nitrilotriacetic acid (NTA) chip consists of a matrix of carboxymethylated dextran preimmobilized with Ni^{2+} (nickel)-NTA. It is used to immobilize naturally and recombinant histidine-tagged molecules.
- Another chip (C1 four-channel sensor chip) was selected for use by Schlecht et al. (2002) to measure two herbicides: 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). This was performed by immobilizing haptens directly on the surface of the chip using a thiol immobilization method.

For analytes with a low molecular weight, it is often necessary to employ an indirect measurement method where the analyte is immobilized directly on a biosensor chip. If the analyte is immunogenic (an antibody can be raised against it), then it is possible to use a competitive or inhibition immunoassay to measure it (Fig. 20.4). An inhibition assay involves the combination of the sample of interest with the specific antibody before injection onto a biosensor chip containing an immobilized target molecule. There is competition

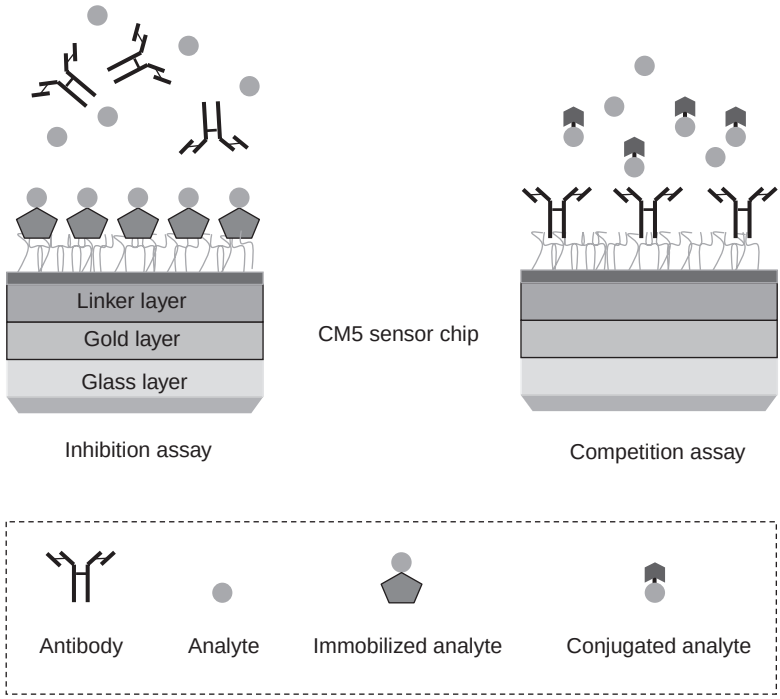


Fig. 20.4 Formats for inhibition and competition assays. *Inhibition*: Free analyte inhibits binding of the antibody to the immobilized analyte on the chip. The signal generated when the antibody binds to the immobilized analyte is inversely proportional to the concentration of free analyte in the sample. *Competition*: Free (●) and conjugated (●) analyte compete for binding to the immobilized antibody. The signal generated is inversely proportional to the amount of free analyte in the sample.

between the immobilized and free antigen for antibody binding. A change in signal is recorded, and this is inversely proportional to the amount of target molecule that remains free in solution. An alternative method to detect analytes of interest involves a competitive assay. In this format, the antibody is immobilized on the surface of a suitable matrix, and the sample is mixed with a standard target molecule that has been conjugated to a large carrier protein. This results in the sample and the conjugated standard competing for the immobilized antibody on the surface of the biochip. An increase in signal is caused by the binding of the large analyte–carrier conjugate, and the data generated is similar to the inhibition assay since the signal recorded is inversely proportional to the amount of target molecule present in the sample.

20.3.1 Immunobiosensors

Many biosensor-based assays have utilized antibodies (immunoglobulins) as the molecular recognition element, leading to the adoption of the term "immunobiosensor." Antibodies are the key recognition elements of the immune system, and various antibodies and antibody-derived fragments have been produced with the capability of detecting a remarkable variety of diverse analytes (Dillon et al., 2005). Antibodies are globular glycoproteins (sugar-containing proteins), with five classes (or serotypes) existing—IgA, IgM, IgE, IgD, and IgG. Single antibody molecules typically have molecular weights of ~150–200 Da. IgG

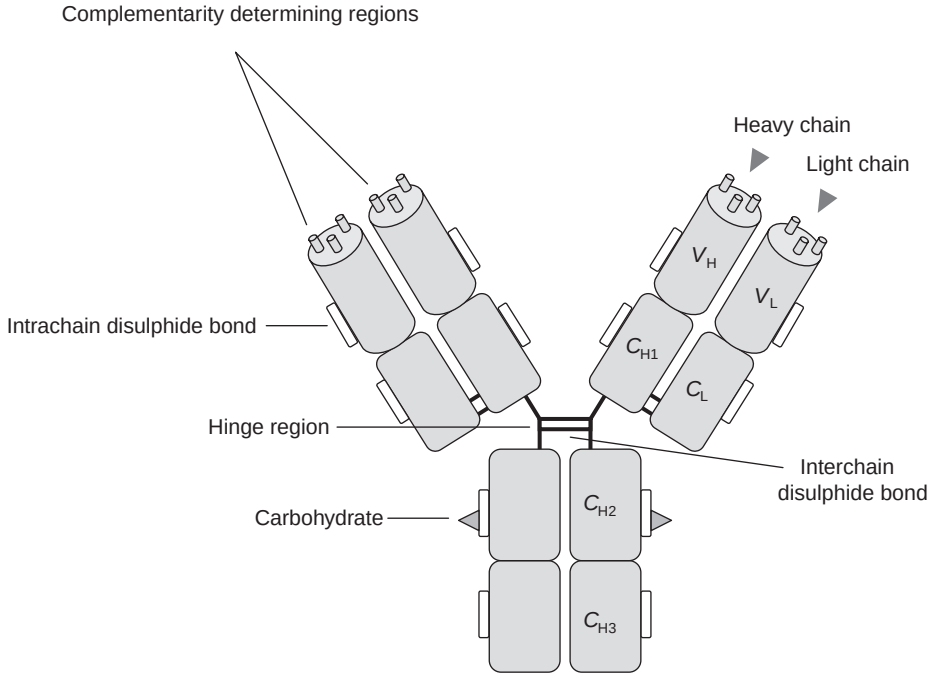


Fig. 20.5 A general schematic diagram of an IgG antibody. The following terms are used in the diagram: V_H, variable heavy region; V_L, variable light region; C_H, constant heavy region; C_L, constant light region. The location of the carbohydrate moiety attached to the C_{H2} constant region is also shown.

antibodies (Fig. 20.5) have a Y-shaped backbone with four polypeptide chains located in two identical chains that are covalently attached through disulfide bonds. The innermost chains are referred to as the heavy chains because they are approximately double the molecular weight of the outer arms (termed the light chains). The recognition sites of the antibody (which interact with an epitope on an antigen) are located at the ends of the V_H (variable heavy) and V_L (variable light) regions of the heavy and light chains. They are commonly referred to as the complementarity-determining regions (CDRs). Each arm of an antibody can bind to one antigen, so one IgG molecule can theoretically bind to two antigens. The strength of an antibody–antigen interaction is referred as the "affinity" of the antibody. This is important when using an antibody in an immunosensor-based system to detect the presence or absence of an antigen in a fruit or vegetable sample.

There are three main methods for generating antibodies that may be implemented in biosensor-based platforms.

The production of polyclonal antibodies involves the immunization of animal hosts to generate an immune response toward a particular antigen. Blood samples are subsequently collected, and the antibodies generated are purified from the serum of the animal. These typically consist of a variety of different serotypes with varying affinities/specificities toward the epitope in question. Any large animal, including guinea pigs, rabbits, goats, sheep, and donkeys, is capable of producing these antibodies (Leenaars and Hendriksen, 2005).

The second method of generating antibodies is by using hybridoma technology to produce monoclonal antibodies (Köhler and Milstein, 1975; Nelson et al., 2000; Hudson and

Souriau, 2003). These are generated by immunizing an animal with the antigen of interest. Once a sufficient immune response is detected, the spleen, bone marrow from long bones (femur and humerus), or primary lymphoid organs (such as lymph nodes) are removed from the sacrificed animal and the antibody-producing B-cells are harvested. These cells can then be fused to immortal myeloma cells (using an electrical current or by using polyethylene glycol). The resulting hybrid cells (hybridomas), which secrete antibodies that are directed toward the desired antigen, are then selected and cloned out to ensure monoclonality. The advantage of this approach is that there is a constant supply of the antibody that is required for analysis. However, there is a significant cost involved in the production and the screening of these antibodies.

Recombinant antibodies, expressed in bacterial strains such as *Escherichia coli*, as well as fungal and mammalian cells, are a very effective alternative. In 2003, 30% of the antibodies used in clinical trials in the biopharmaceutical industry were recombinant (Hudson and Souriau, 2003). These antibodies are employed in a phage-display format (Bradbury and Marks, 2004).

The ability of molecular biology to increase the affinity of an antibody for an antigen has permitted the use of a variety of high-affinity antibodies in biosensor-based platforms. The amino acid sequence of a CDR region may be altered to enhance the binding characteristics of an antibody by site-directed mutagenesis. Furthermore, antibody fragments (Fig. 20.6) may be generated through the enzymatic digestion of an antibody by papain, which targets

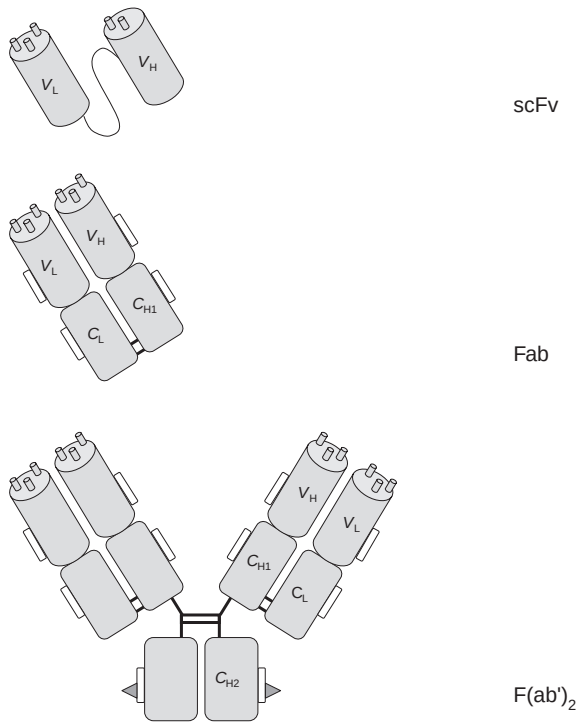


Fig. 20.6 Different antibody fragments that may be used for biosensors. The F(ab′)$_2$ is obtained through papain digestion. The Fab and scFv are derived through recombinant techniques.

the hinge region of an antibody, resulting in two identical Fab (fragment antigen binding) fragments. The presence of the constant regions in a Fab is thought to aid in the stabilization of the antibody variable regions, which might not function efficiently when expressed in the monomeric single-chain fragment variable (scFv) format (Röthlisberger et al., 2005). It was recently shown in our laboratory that the Fab antibody format is the most reliable and sensitive format for use in small molecule competition biosensor assays. The strict monovalency of this format can lead to a significant enhancement in assay sensitivity in both ELISA and competition SPR analyses (Townsend et al., 2006).

The scFv is the most widely used antibody fragment. The variable regions (V_H and V_H) of the antibody are linked by a flexible peptide linker. The most frequently used linkers are based on glycine-serine repeat structures and can be of different lengths. The length of the linker is related to the intended valency of the molecule. When a short linker is used, the stability and folding of the scFv do not occur properly. This is caused by the insufficient juxtaposing of the V_H and V_L regions in the single chain for the monomer to function. ScFvs selected in this format invariably form bivalent dimers as a result (Holliger et al., 1993; Kortt et al., 1997; Atwell et al., 1999), or "diabodies," which often have increased avidity for an antigen over the monomeric forms typically observed when long-linker systems are used. Long linkers (ranging from 18 to 21 amino acids) favor the production of scFv formats, which are predominantly monomeric (Holliger et al., 1993; Perisic et al., 1994; McGuinness et al., 1996). The incorporation of these fragments into antibody-based assay formats is important for providing sufficient sensitivity for a vast range of diagnostic approaches that could be applied in the food industry (Hudson and Souriau, 2003).

There have been several excellent examples of biosensor-based analysis of fruit and vegetable products, and some of the more interesting observations are now discussed. Minunni and Mascini (1993) used Biacore to detect traces of the herbicide atrazine in water samples by immobilizing an atrazine conjugate onto the surface of the sensor buffer containing a known amount of free antibody and the herbicide analyte. This competitive assay yielded a detection limit of 50 pg/mL. Moran et al. (2002) used SPR to characterize antibodies that were used to detect the presence of 2-(4-thiazolyl)benzimidazole, a molecule which is used as a food preservative and an agricultural fungicide. Caldow et al. (2005) used a Biacore Q instrument to detect the bacteriostatic antibiotic tylosin in bee's honey. This polyketide is active against most gram-positive bacteria, mycoplasma, and certain gram-negative bacteria. They were able to detect tylosin at the level of 2.5 μg/kgin honey. Finally, Schlecht et al.(2002) used a C1 four-channel sensor chip in a study to quantifiably detect the presence of two structurally similar organochlorine herbicides, namely, 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). This was achieved by immobilizing 2,4-D analogs onto the surface of the C1 chip through a thiol-carboxyl group reaction, and this enabled 2,4-D to be quantified down to a concentration of 0.1 μg/mL.

20.4 Electrochemical sensors

Electrochemical sensors have also been used extensively to detect analytes of interest in agricultural produce. The biorecognition element in these sensors is in direct contact with a transducer, and the resulting signal that is generated is converted from a biochemical signal to an electrical signal. The biorecognition elements incorporated into these devices can

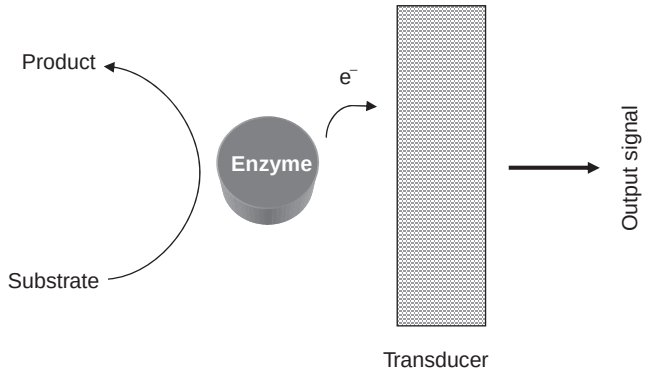


Fig. 20.7 Schematic diagram of an enzyme transducer biosensor. When the redox enzyme goes through its catalytic cycle (going from an oxidized to reduced state and back to its resting state), the redox action of the enzyme is detected by the transducer and the change in electrical state is recorded as a change in the output signal. An electron is represented by e^-.

consist of enzymes, antibodies, and other cellular components. Enzymes that belong to the oxidoreductase class (enzyme classification EC 1) are commonly selected for use because they alternate between oxidized and reduced states, which can be measured electrochemically, and can therefore be exploited in analytical devices. Equation (20.1) illustrates the generation of an electron through the redox cycling of an enzyme. When the enzyme is located in close proximity to the surface of the transducer, electron transfer can occur directly (as shown in Fig. 20.7). However, as is the case with many naturally occurring enzymes, they are surrounded by a layer of carbohydrate or lipid. This increases the distance that electrons have to traverse, thus causing a decrease in the signal recorded by the transducer. In situations where this occurs, electron mediators are used (Fig. 20.8). These mediators are compounds that are also electroactive, and a common example of an electron mediator is ferrocene. Table 20.5 illustrates the characteristics that are favorable in choosing a specific mediator (Cassidy et al., 1998). Equation (20.2) illustrates the reaction of the mediator and the subsequent generation of an electron resulting from the electrochemical cycling of the

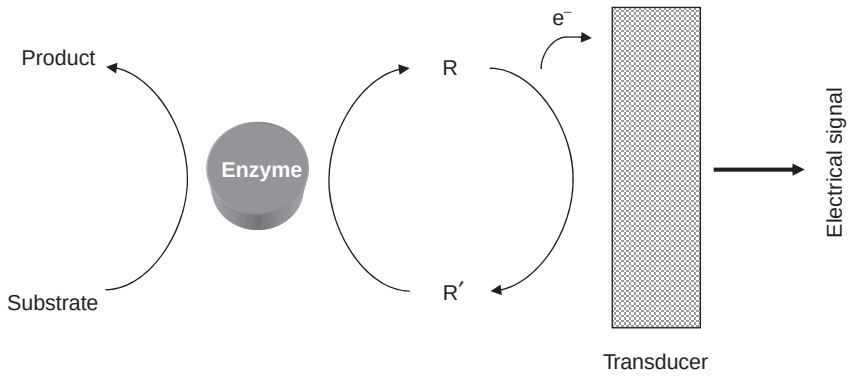


Fig. 20.8 An enzyme-based electrochemical biosensor with an electron mediator. The mediator shuttles the electron (e^-) from the enzyme to the surface of the transducer where it is converted from a chemical signal to an electrical signal. R and R′ represent the oxidized and reduced forms, respectively, of an electron mediator.

Table 20.5 The ideal characteristics and properties of an electron mediator

1.	Exhibits reversible kinetics
2.	Reacts readily with the reduced form of an enzyme
3.	Has a low oxidation potential and is pH independent
4.	Is stable in both redox forms
5.	Easily retained at the surface of an electrode
6.	Unreactive toward oxygen
7.	Chemically unreactive with the immobilized biological material

mediator. Mediators are usually low molecular redox couples that shuttle the electrons from the enzyme's active site to the surface of the transducer.

$$H_2O_2 + 2H^+ + 2e^- \longrightarrow 2H_2O \tag{20.1}$$

$$\text{Mediator}_{red} \longrightarrow \text{Mediator}_{ox} + e^- \tag{20.2}$$

Equations (20.3)–(20.6) illustrate a peroxidase-mediated reaction on a biosensor surface. Equation (20.3) shows the resting state peroxidase (in its reduced form) reacting with hydrogen peroxide and two hydrogen ions to form an intermediary oxidized-peroxidase compound and two water molecules. Equation (20.4) shows the reaction of the intermediary oxidized-peroxidase compound with the reduced form of a mediator to produce the resting reduced-state peroxidase and an oxidized mediator. Equation (20.5) shows the oxidized mediator reacting with two electrons, thus reverting to the reduced form of the mediator. It is these electrons that the transducer detects and converts into an electrical signal. The final Eq. (20.6) shows the overall reaction, whereby one hydrogen peroxide molecule in the presence of two electrons and two hydrogen ions is converted to two water molecules (Ryan et al., 2006).

$$H_2O_2 + 2H^+ + PO_{red} \longrightarrow PO_{ox} + 2H_2O \tag{20.3}$$

$$PO_{ox} + \text{Mediator}_{red} \longrightarrow PO_{red} + \text{Mediator}_{ox} \tag{20.4}$$

$$\text{Mediator}_{ox} + 2e^- \longrightarrow \text{Mediator}_{red} \tag{20.5}$$

$$H_2O_2 + 2e^- + 2H^+ \longrightarrow 2H_2O \tag{20.6}$$

It is often possible to use a multienzyme electrochemical biosensor system to detect the presence and determine the concentration of a particular compound in a material. This method has two or more enzymes in proximity with each other and in contact with a mediator or directly in contact with the transducer. As seen in Fig. 20.9, enzyme 1 (glucose oxidase) generates H_2O_2 as a byproduct by the catalysis of glucose by glucose oxidase. The H_2O_2 generated is catalyzed by a peroxidase enzyme and the redox cycling of the mediator. The cycling of the peroxidase enzyme in the presence of H_2O_2 generates electrons that are

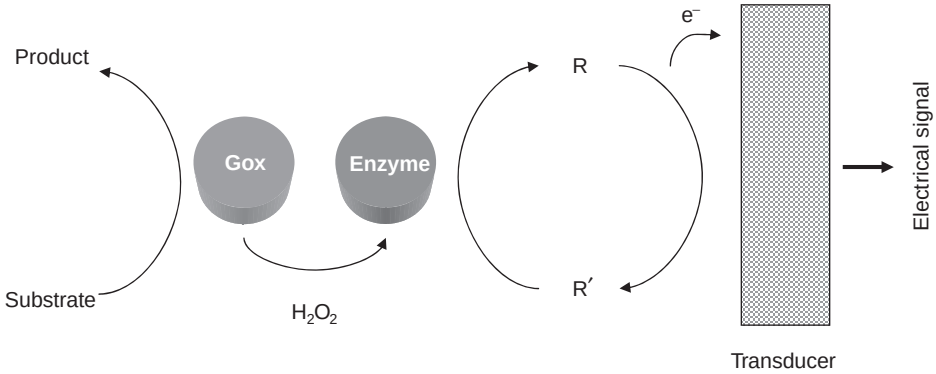


Fig. 20.9 A representation of a sensor with two enzymes operating together. The first enzyme catalyzes the substrate of interest and, as a byproduct, H_2O_2 is generated. This is then catalyzed by a peroxidase enzyme. The redox activity of the peroxidase enzyme is detected by the transducer. Gox is glucose oxidase; H_2O_2 is hydrogen peroxide; R and R′ represent the oxidized and reduced forms, respectively, of an electron mediator; and e^- is an electron.

detected by the transducer. This in turn enables the concentration of the target analyte to be determined.

There are several well-characterized electrochemical biosensor devices that have been applied in the fruit and vegetable industry for the detection of pesticides, herbicides and insecticides, organophosphates, organochlorines, and carbamates. There are many different ways to detect the presence of a pesticide. One of these protocols involves the incorporation of enzymes, which are directly affected by the presence of the pesticide, into an analytical platform. Acetylcholinesterase (AChE), an enzyme present in muscles, red blood cells, and nerve tissue that catalyzes the hydrolysis of the neurotransmitter acetylcholine to yield choline and acetic acid, is associated with cognition in mammalian hosts. Compounds that effect AChE activity include organophosphates and parathion. Botulinum toxin, from the bacterial strain *Clostridium botulinum*, also suppresses the release mechanism of AChE (Rang et al., 1998). The suppression of activity of this enzyme results in the accumulation of acetylcholine in the host, which can cause an excessive overstimulation of the cholinergic nerves. Death usually results from the failure of the respiratory and circulatory systems (Timbrell, 1991). Schulze et al. (2002) developed an amperometric AChE biosensor that was used for the detection of several carbamates and organophosphates in a number of fruit products that were sampled from a variety of countries. The protocol involved the printing of thick film electrodes onto sheets of polyvinylchloride and "curing" for 30 min at 90°C prior to the introduction of AChE by glutaraldehyde coupling. The activity of AChE was analyzed by monitoring the formation of thiocholine by the enzymatic hydrolysis of acetylcholine chloride. This sensor format permitted the detection of trace levels of these analytes (lower than 5 μg/mL), and the major benefit of this protocol is a reduced analysis time in comparison to conventional methods.

Other examples of how electrochemical biosensors have been used to monitor the presence of "indicator molecules" include the following.

Kriz et al. (2002) used a SIRE technology–measuring principle (sensors based on injection of the recognition element) to monitor L-lactate content in baby food and tomato paste,

whereby a small amount of enzyme (lactate oxidase) was injected into an internal delivery flow system and was held in direct spatial contact with an amperometric transducer by a semipermeable membrane. Measurements were determined by the enzymatic conversion of L-lactate to pyruvate and H_2O_2. This method could detect concentrations of L-lactate in the range of 0.10–2.51 mM. All assay measurements were compared to an established spectrophotometric assay. However, the authors state that the biosensor method has a distinct advantage as the measurement can be performed in less than 3 min compared to 30–35 min for the spectrophotometric method.

Voss and Galensa (2000) used an electrochemical detection protocol to monitor the presence and concentration of amino acids in fruit juice. Amino acids were separated on a lithium cation–exchange column, and an amperometric biosensor was used to monitor the production of H_2O_2. This protocol could detect concentrations ranging from 0.1 mg/mL to 5 mg/mL (D-Pro and D-Met to L-Ala, respectively). D-Alanine could be detected in concentrations of 0.5 mg/mL. In this case, the presence of D-alanine may be indicative of bacterial contamination.

Wheat is a universal component of food produce, and the monitoring of pesticide residues in this product is of critical importance. Del Carlo et al. (2005) quantified the amount of phosphothionate insecticide (pirimiphos-methyl) present in durum wheat using an electrochemical biosensor. They used an AChE-inhibition assay and obtained a calibration curve between 25 and 1,000 ng/mL with a detection limit of 38 ng/mL. When real samples of durum wheat were analyzed, the limit of detection increased to 65–133 ng/mL because of the sample matrix. In EU, regulations the pirimiphos-methyl MRL is 5 mg/kg.

A comprehensive review paper has been published that examines biosensors that use enzyme inhibition to measure the presence of pollutants and toxic compounds in food samples (Amine et al., 2006). Other types of biosensors have also been used for postharvest analysis. Pogačnik and Franko (2003) developed a photothermal biosensor to measure organophosphate and carbamate compounds in salads, lettuce, and onions. This approach used thermal lens spectrometry, a technique that depends on the adsorption of optical radiation in the sample, generating heat. This introduces a change in the RI, and through this analysis, concentrations can be determined. Paraoxon was detected in all of the samples tested by this protocol. Another optical biosensor was developed to detect and quantify carbamate residues in vegetables. It was observed that changes in the concentration of carbamate could be monitored using chlorophenol red (Xavier et al., 2000).

Keay and McNeil (1998) developed a protocol that utilized a competitive ELISA that exploits a disposable screen-printed horseradish peroxidase (HRP)-modified electrode as the detector element in tandem with a single-test immunomembrane to measure traces of atrazine. A monoclonal antibody for atrazine was immobilized onto a membrane that was subsequently placed onto the electrode surface, and the assay used a glucose oxidase label attached to the atrazine molecule. The quantification of atrazine in solution was permitted by the competition between labeled and unlabeled atrazine for binding to the immobilized antibody.

Another novel approach for the detection of atrazine involved the incorporation of liposomes (small lamellar vesicles) into a competitive assay format. One of the advantages over other formats, including disposable immunostrip assays, was the fast turnaround time for analysis (7 min compared to 20 min for the immunostrip assays) (Bäumner and Schmid, 1998). Concentrations of 0.1 μg/L of atrazine could be detected with this system. In another

electrochemical method, López et al. (1998) used a redox polymer that was coimmobilized with an antibody embedded in hydrogel on the surface of an electrode to detect atrazine. The assay format was competitive and incorporated a HRP-labeled antigen. They achieved detection limits of 1 ppb (part per billion).

Electrochemically based assay formats may be further enhanced by electrochemiluminescence (ECL). ECL reactions are of interest because of their versatility for a range of different types of immunoassay. The principle of ECL is the cooxidation of luminol and a substrate (called an enhancer) by hydrogen peroxide in the presence of the enzyme HRP. The resulting amperometric signal is detected by the transducer, and an electrical signal is generated. ECL as a detection system has advantages over other methods, including high sensitivity and a reduced assay time. In experiments carried out by Rubtsova et al. (1998), specific antibodies against atrazine were covalently immobilized on photoactivated nylon. A chemiluminescence-based assay was then used to measure the herbicide 2,4-D with a detection limit of 0.2 μg/L.

20.5 Biosensor arrays

In recent years there is a steadily increasing trend for the development of biosensors in an array format, where multiple analytes can be measured simultaneously on a single device. An enzyme-based three-electrode biosensor for detecting the presence of markers of maturity and quality in tropical fruits was developed by Jawaheer et al. (2003) to measure the analytes β-D-glucose, total D-glucose, sucrose, and ascorbic acid. These markers are indicative of maturity and quality in selected fruit products and were assayed in pectin (a natural polysaccharide present in plant cells). A fabrication format was developed that permitted the integration of the individual sensors into a multisensor array, and analytes were measured in this matrix to enhance the enzymatic responses over analyte ranges of 0–7 mM. Interferences normally related to electrochemically active compounds present in fruits were minimized by including a membrane made out of cellulose acetate.

Several bacterial strains are of great significance for the horticultural industry. Pathogenic bacteria in food account for 90% of food-borne illnesses in the United States. The bacterial strain *Escherichia coli* 0157:H7 is a major pathogen of interest in fruits and vegetables as it causes severe illness and can be fatal in infants, the elderly, and the immunocompromised. A disposable conductometric electrochemical biosensor, based on a lateral flow strip connected to an ohmmeter, was described by Muhammad-Tahir and Alocilja (2004) to detect this strain and *Salmonella* spp. Anti-*E. coli* 0157:H7 antibodies, labeled with polyaniline, were immobilized onto the nitrocellulose strip, and a sample was allowed to migrate up the strip. A drop in resistance, proportional to the concentration of *E. coli* 0157:H7 cells binding to the antibodies, indicated a decrease in the electron transfer from the polyaniline-conjugated antibody.

Listeria monocytogenes is a highly infectious pathogen that has very serious implications if present in food or food products due to the associated risk of fatality. While the natural ecosystem of this bacterial strain includes soil, water, plant material, and decaying plant detritus (Suihko et al., 2002), numerous foods, such as raw vegetables, fruits, and horticultural samples, are also prone to infection. The psychrophilic nature of this strain, conferring the ability to grow at refrigeration temperature, and the ability of this bacterium to withstand high salt concentrations and tolerate a wide pH range suggest that

Table 20.6 Examples of SPR analysis of contaminants commonly found in fruit and vegetables

Analyte	Limit of detection	Reference
Salmonella enteritidis, Listeria monocytogenes	10^6 cells/mL	Koubová et al. (2001)
Salmonella groups B, D, and E	1.7×10^3 cfu/mL	Bokken et al. (2003)
Staphylococcal enterotoxin B	0.5 ng/mL	Homola (2003)
Staphylococcal enterotoxin B	1.0 ng/mL	Nedelkov et al. (2000)
Salmonella typhimurium	10^2–10^9 cfu/mL	Oh et al. (2004)
Atrazine	0.5 mg/mL	Minunni and Mascini (1993)
Atrazine	1–100 mg/mL	Nakamura et al. (2003)
2,4-D, 2,4,5-T	0.1 mg/mL	Schlecht et al. (2002)

contamination of food is a frequent occurrence. Leonard et al. (2005) developed a rapid SPR-based detection method for the detection of *L. monocytogenes*. A polyclonal antibody was generated to detect Internalin B (InlB), a protein on the surface of *L. monocytogenes* that participates with Internalin A (InlA) in the invasion of mammalian cells. An inhibition assay was then used to monitor the presence of cells in solution. This involved the incubation of the specific polyclonal antibody with varying concentrations of cells. They were then injected over a CM5 sensor chip with immobilized anti-InlB antibodies. A decrease in the antibody binding of InlB when increasing concentrations of *L. monocytogenes* cells were present in the original sample was observed, and a detection limit level of less than 10^6 cells/mL of sample was reported. Hearty et al. (2006) generated a monoclonal antibody that specifically interacted with the InlA surface protein and showed that this antibody could be used to detect *Listeria* InlA using a Biacore-based assay. Tully et al. (2005) demonstrated the use of quantum dot-labeled antibodies specific for InA for the immunostaining of *L. monocytogenes* cells, and these have major potential for use with fluorescence-based sensor formats. Table 20.6 shows further examples of the use of SPR instruments to detect and measure the presence of bacteria and pesticides commonly found in fruit and vegetables.

Mycotoxins are toxic compounds that are synthesized as secondary metabolites by fungal strains. The fungus *Fusarium moniliforme* produces the carcinogenic mycotoxin, Fumonisin B_1. This is one of a number of major fungal species that can infect corn (http://fumonisin.noneto.com/). Mullett et al. (1998) developed an SPR method to detect this mycotoxin by immobilizing varying concentrations of Fumonisin B_1-specific antibodies onto a gold layer and then passing a standard solution of Fumonisin B_1 over the surface prior to measuring the change in SPR. Their assay had a detection limit of 50 ng/mL. This research group states that the homemade SPR device used in their research could be implemented as an early-stage method in the screening of large numbers of potentially contaminated food samples. Positive samples could then be analyzed further by more elaborate methods of analysis, such as liquid chromatography/electrospray ionization mass spectrometry (Hartl and Humpf, 1999).

The monitoring of the presence of aflatoxins, which are naturally occurring mycotoxins produced by several strains of *Aspergillus* spp., in fruit, vegetable, and food produce is of great significance. Produce that is prone to contamination includes nuts (almonds, walnuts), cereals (rice, wheat, maize), and oilseeds (soybean and peanuts). Aflatoxin monitoring is also important as consumption of infected produce can manifest in carcinoma of the liver (Bhatnagar and Ehrlich, 2002; Bennett and Klich, 2003). While approximately 16

structurally diverse aflatoxins have been reported, aflatoxins B_1, B_2, G_1 and G_2, and M represent the greatest danger to human health (Keller et al., 2005). All of these compounds may be assembled in *Aspergillus parasiticus*, while *Aspergillus flavu* can synthesize aflatoxins B_1 and B_2 independently (Yu et al., 2004).

Due to the agricultural importance of these mycotoxins, several biosensor-based platforms have been developed to permit the detection of trace levels of different aflatoxins (Lacy et al., 2006). Several of these protocols have been carried out in our laboratory and have focused on aflatoxin B_1 (AFB_1). Daly et al. (2000) used a rabbit-derived polyclonal antibody to detect AFB_1, which was conjugated to BSA and immobilized onto a CM5 Biacore chip. A competition assay between free and bound AFB_1 permitted a linear range of detection of trace levels (3–98 ng/mL). Daly et al. (2002) subsequently generated murine scFvs against AFB_1 by using a phage-display format and incorporated these antibodies into a Biacore-based inhibition assay. Dunne et al. (2005) developed a unique SPR-based inhibition assay that incorporated monomeric and dimeric scFv antibody fragments that could detect AFB_1 immobilized on a CM5 Biacore chip. Monomeric scFvs could detect between 390 and 12,000 ppb, while the dimeric scFv was more sensitive, detecting between 190 and 24,000 ppb.

Several other research groups have developed similar rapid analytical biosensor-based platforms for aflatoxin detection. Carlson et al. (2000) developed a handheld biosensor to detect minute traces of aflatoxins at concentrations of between 0.1 and 50 ppb. In another elaborate experiment, Sapsford et al. (2006) developed an indirect competitive immunoassay on a fluorescence-based biosensor that permitted rapid detection of AFB_1 in spiked corn (cornflakes, cornmeal) and nut (peanuts, peanut butter) products. Mouse monoclonal antibodies were labeled with the fluorescent dye CY5, with detectable signals inversely proportional to the concentration of AFB_1 present. Limits of detection for nut and corn products were 0.6–1.4 and 1.5–5.1 ng/g, respectively. Adanyi et al. (2007) also recently described a unique protocol for permitting aflatoxin detection by using optical wavelength light mode spectroscopy (abbreviated to OWLS). Integrated optical wavelength sensors were selected for use in this experiment with the sensitive detection range for a competitive assay being between 0.5 and 10 ng/mL. An indirect screening protocol was subsequently applied to wheat and barley that permitted the detection of AFB_1 and ochratoxin A.

20.6 Legislation

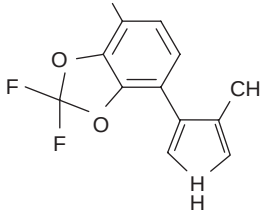
This chapter has discussed a variety of different biosensor-based platforms that may be used to detect a range of different molecules such as pesticides, herbicides, and mycotoxins. It is important to consider the legislation associated with the monitoring of agricultural produce. The Food and Agriculture Organization of the United Nations (FAOSTAT) (http://faostat.fao.org/) is an international body whose overall aim is to protect the health of consumers. FAOSTAT has an online database (The Codex Alimentarius: Pesticide Residues in Food Maximum Residue Limits, see additional websites of interest), which details the maximum residue limits (MRLs) for pesticides in commodities such as fruit and vegetables. The establishment of an MRL (usually expressed as mg/kg) is dependent on good agricultural practice for pesticides. This relates to where the highest detectable residues anticipated (when a pesticide-containing product is applied to a commodity to remove contaminants) are intended to be toxicologically acceptable. Under these arrangements, the important

point is that residue levels do not pose unacceptable risks for consumers by being present in values that exceed these levels.

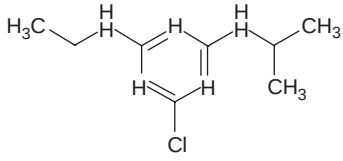
Similar legislation exists within the European Union. Within the EU Directive (91/414/EEC), the Plant Protection Products Directive (The Authorisations Directive: 1991) was proposed with the aim of harmonizing the overall arrangements for authorization of plant protection products within the EU. However, legislation relating to MRLs of pesticides in cereals, fruits, vegetables, foods of animal origin, and feeding stuffs was substantially amended several times since this legislation was developed. A single act has replaced the amended original Directive Regulation (EC) No. 396/2005. This establishes maximum levels of pesticide residues permitted in or on food and feed of plant and animal origin. These MRLs include levels that are specific to particular foodstuffs that are intended for human or animal consumption, and a general limit that applies where no specific MRL has been established. Finally, as infants have underdeveloped immune systems, fruit and vegetable-based baby foods need to be rigorously monitored, as the presence of low concentrations of pesticides could invariably be fatal. The EU has set an MRL for any given pesticide in infant foods of a concentration not exceeding 10 μg/mL. This information is very significant for the use and future applications of biosensor-based detection methods.

20.7 Appendix

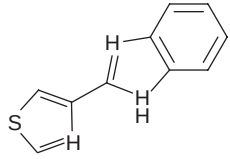
This illustrates some of the compounds described in the text that are of relevance to the fruit and vegetable industry.



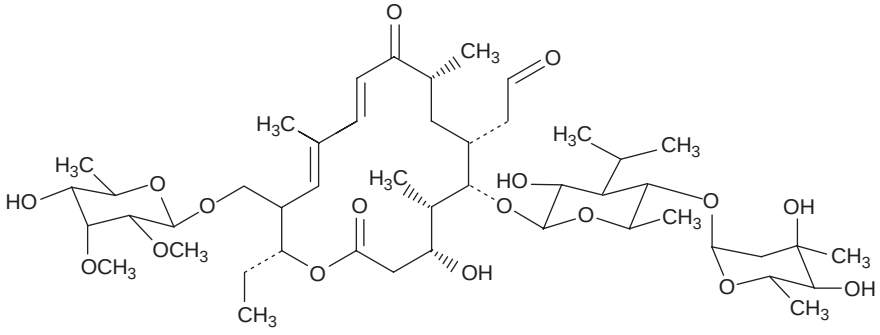
Fludioxonil



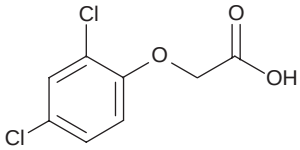
Atrazine



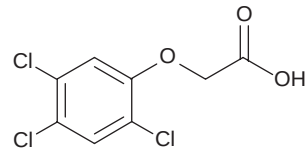
2-(4-Thiazolyl)benzimidazole



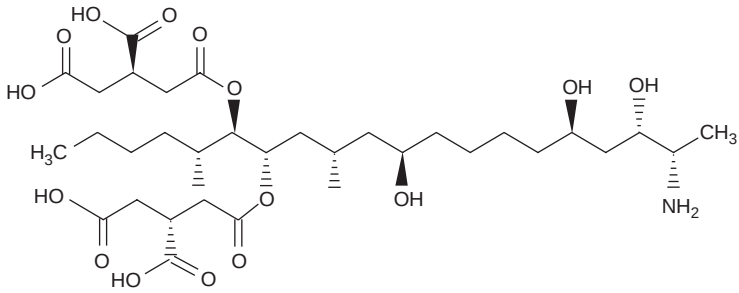
Tylosin



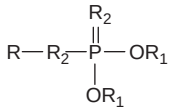
2,4-Dichlorophenoxyacetic acid



2,4,5-Trichlorophenoxyacetic acid

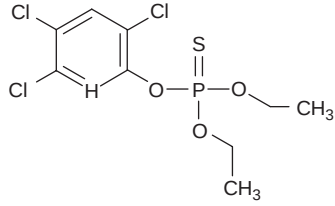


Fumonisin B1

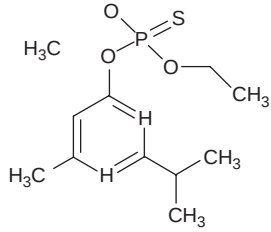


General organophosphate structure

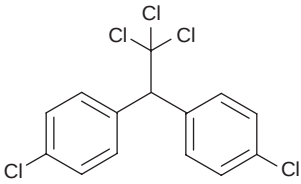
R_1 – CH_3 or CH_2CH_3
R_2 = O or S;



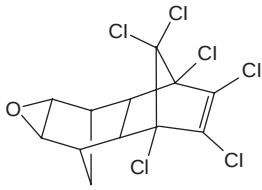
Chlorpyrifos (organophosphate)



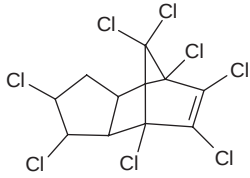
Diazinon (organophosphate)



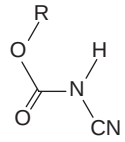
Dichloro-diphenyl trichloroethane DDT (organochloride)



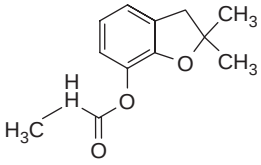
Dieldrin (organochloride)



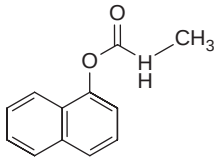
Chlordane (organochloride)



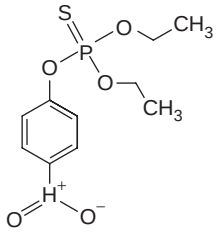
General carbamate structure



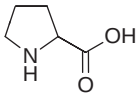
Carbofuran (carbamate)



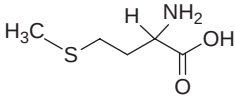
Carbaryl (carbamate)



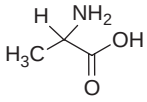
Parathion



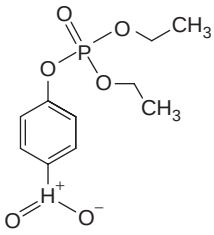
D-Proline



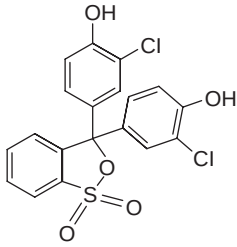
D-Methionine



D-Alanine

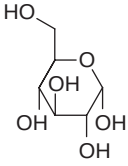


Paraoxon

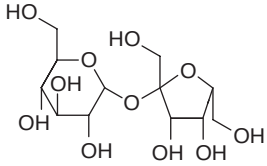


Chlorophenol red

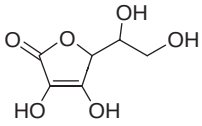
Triazine



D-Glucose

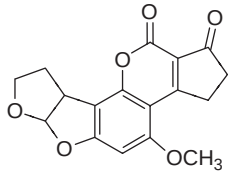


Sucrose

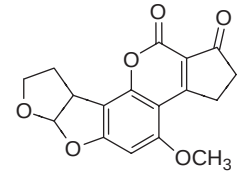


Ascorbic acid

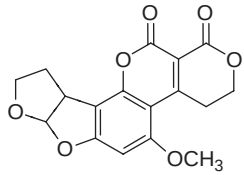
Ferrocene



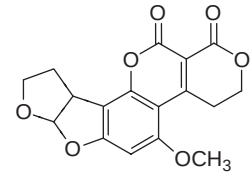
Aflatoxin B_1



Aflatoxin B_2



Aflatoxin G_1



Aflatoxin G_2

Acknowledgments

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Additional websites of interest

Food and Agriculture Organization of the United Nations (Homepage): http://www.faostat. fao.org/

Pesticide Residues in Food (MRLs/EMRLs): http://www.codexalimentarius.net/mrls/pestdes/jsp/pest_q-e.jsp?language=EN&version=ext&hasbulk=0

The Pesticides Safety Directorate (Homepage): http://www.pesticides.gov.uk/

Fumonisin Facts Sheet (Homepage): http://www.fumonisin.noneto.com/

Chapter 21

Changes in Nutritional Quality of Fruits and Vegetables During Storage

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21.1 Introduction

Fruits and vegetables are very important in our day-to-day living. They are valuable sources of vitamins, minerals, fibers, and antioxidants, which are essential for a healthy and well-balanced diet. Consumers prefer to buy fruits and vegetables of high quality based on their appearance (color), sensory quality (texture and taste), and nutritive values. However, fruits and vegetables are highly perishable commodities. As well, recent increase in the consumer demand for fresh-cut fruits and vegetables has increased the industry considerably. Just as in fresh fruits and vegetables, during postprocessing storage, fresh-cut materials are subjected to additional stress factors such as wounding and pathogen infection. In all these situations, the nutritional quality of the produce or products may change, sometimes negatively (Gil et al., 2006).

Fruits and vegetables have a high market value, and the maintenance of quality after harvest is an important issue to growers. Several factors, such as environmental conditions, cultivars, cultural practices, susceptibility to pests and diseases, time of harvest, and postharvest conditions, determine the quality of these commodities. As quality greatly affects the consumers' preferences, it is a key factor for the marketing of fresh fruits overseas. To gain the best value for growers, storage operators, and consumers, it is very important to maintain quality throughout preharvest development, postharvest storage, and subsequent distribution and marketing chains.

During ripening, several biosynthetic pathways contribute to the development of organoleptic qualities in fruits. An ideal quality development in fruit is contributed by the degradation of starch into sugars and that of cell wall, as well as the biosynthesis of several secondary metabolites, which provide color and flavor to the fruits. The developments of ideal color, flavor, sugar levels, and optimal firmness are key parameters that provide satisfactory fruit quality.

21.2 Ripening and softening of fruits

Ripening of fruits is a complex process. A series of physiological, biochemical, and organoleptic changes occur during ripening that transform an inedible fruit into an edible

fruit with optimal quality features. A number of biochemical changes, such as the biosynthesis of anthocyanins, degradation of chlorophyll, enhanced activity of cell wall–degrading enzymes, evolution of aroma compounds, and an increase in respiration and energy production, occur during the progression of ripening (Paliyath and Murr, 2006). Ripening initiates the catabolic breakdown of starch releasing sugars, and organic acids are converted back to sugars through a process called gluconeogenesis.

Fruit softening is an integral part of the ripening, which is associated with textural changes. It involves the alteration in structure and composition of cell wall, degradation of cellulose and pectin components, and breakdown of starch. Depending on the fruit, the nature of softening may vary from one to another. Fruits, including cherries, grapes and banana, undergo extensive fruit softening, whereas extensive softening is not a part of fruit ripening in apple and citrus (Seymour et al., 1993). The cell wall is mainly made of cellulose, pectin, and hemicelluloses. Several enzymes, such as cellulase or β-1,4-glucanase, β-galactosidase, pectin methylesterase, and polygalactouronase, are involved in degradation of cell wall components. It has been found that polygalactouronase activity is lower in cherries than other fruits. Its activity increases during maturation and storage of cherries (Barrett and Gonzalez, 1994). Excessive ripening of the fruit can be reduced by application of calcium. It binds and cross-links with free carboxyl groups of polygalacturonic acid in pectin, which enhances firmness of the fruits (Paliyath and Murr, 2006).

During senescence, phospholipid content of the cell membrane declines with an associated increase in the levels of neutral lipids such as diacylglycerols, free fatty acids, and fatty aldehydes. Moreover, the levels of sterols also increase during senescence. Hence, there is an increase in the ratio of sterol/phospholipids. These changes decrease membrane fluidity ultimately resulting in the loss of cellular compartmentalization, and subsequently leading to senescence (Paliyath and Droillard, 1992). Membrane lipid degradation is initiated by the enzyme phospholipase D (PLD), which liberates phospholipid head groups (choline, ethanolamine, inositol, etc.), and is followed by the activity of enzymes such as phosphatidate phosphatase, lipolytic acyl hydrolase, and lipoxygenases. In this process, peroxidized fatty acids produce compounds like hexanal and hexenal, which are important fruit volatiles. The short-chain fatty acids undergo β-oxidation and form short-chain fatty acyl CoAs and fatty alcohols. These products are esterified enzymatically by alcohol—acyl coA acyl transferase, giving rise to the volatile flavor components in fruits (Paliyath and Droillard, 1992).

21.3 Role of ethylene in ripening

The process of ripening is initiated by the plant hormone ethylene. On the basis of ethylene production and response to externally applied ethylene, fruits can be classified into climacteric and nonclimacteric. Climacteric fruits display a burst in ethylene production and respiration during ripening. In these fruits, synthesis of ethylene is autocatalytic, which can reach tissue internal levels of 30–500 μL/L or more. However, nonclimacteric fruits, such as cherries, strawberries, and grapes, neither require a high level of ethylene during the initiation of ripening nor do they produce ethylene autocatalytically as in climacteric fruits. The rate of ethylene biosynthesis is also influenced by several external factors that mainly include storage temperature and the levels of O_2 and CO_2 during postharvest storage. It has

been found that in some temperate fruits such as pears, ethylene synthesis can be induced by low temperature.

The precursor for ethylene biosynthesis is methionine, which is converted to *S*-adenosyl methionine (SAM) in the presence of the enzyme methionine adenosyl transferase. Next, the enzyme ACC synthase converts SAM into 1-aminocyclopropane-1-carboxylic acid (ACC), which is the immediate precursor of ethylene. The enzyme 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) converts ACC into ethylene. At low temperature, accumulation of the enzyme ACO is induced (Lelièvre et al., 1997). During modified and controlled atmosphere (CA) storage, the level of oxygen also affects the rate of ethylene production. At low levels of oxygen (1–3%), ethylene production is reduced because oxygen is a co-substrate for ACO (Lelièvre et al., 1997). Studies have demonstrated that ethylene controls several organoleptic quality changes that occur during ripening (Tian et al., 2000; Zhu et al., 2005; Gao et al., 2007). Binding of ethylene to its receptors accelerates the process of fruit ripening and senescence. It has been proposed that after binding to its receptors, ethylene releases calcium from storage compartments, which initiates phospholipid degradation by binding of PLD to plasma membrane (Pinhero et al., 2003).

Several methods have been developed for the inhibition of ethylene biosynthesis and its action. Aminoethoxyvinylglycine (AVG), marketed commercially as ReTain®, is an inhibitor for ACC synthase, which is an important step in ethylene biosynthesis. Delayed fruit ripening and reduced fruit drop was noted in AVG-treated apples and pears (Rath et al., 2006). 1-Methylcyclopropene (1-MCP) inhibits ethylene action by preventing its binding to the ethylene receptors, which enhances fruit shelf life and firmness. It is marketed commercially as SmartFresh™ and is used for postharvest treatments in apples and tomatoes. This treatment is effective at low concentration, and no residue has yet been detected in treated fruits. Its affinity to the receptors is 10-fold greater than that of ethylene (Blankenship and Dole, 2003). The use of 1-MCP for different horticultural crops has been approved in several countries.

The optimally effective concentration and exposure time for 1-MCP vary with the commodity and treatment temperature. For apples, the effective concentration to delay ripening is 1 ppm. It has been reported that exposure to a lower concentration of 1-MCP for a longer duration has the same effect as the exposure to a higher concentration for a short duration. In cut carnations, the effect of exposing to 250–300 nL/L of 1-MCP for 5 min was the same as that of an exposure to 0.5 nL/L for 24 h (Blankenship and Dole, 2003). It has been observed that the effect of 1-MCP in preventing loss of fruit firmness is decreased at lower temperature. This could be because of lower affinity of 1-MCP binding at low temperature (Blankenship and Dole, 2003). It was noted that apples at 3°C required 9 h of 1-MCP treatment to maintain fruit quality during prolonged CA storage; however, this time requirement was reduced at higher temperature. Moreover, treatment time and temperature also depend on cultivars. In order to get the same physiological effects of 1-MCP at the same concentration, "Empire" apples needed less treatment time than "Cortland" apples (DeEll et al., 2002).

21.4 Fruit quality enhancing metabolic pathways

Several metabolic pathways are involved in the synthesis of components that determine the organoleptic qualities during fruit ripening. Respiration is increased during ripening, which

provides energy (ATP) for metabolic processes. In general, starch and organic acids are the main storage components in fruits. With the advancement of ripening, starch is metabolized to sugars, which are also synthesized through gluconeogenesis giving rise to sweetness in the fruits. The fruits develop attractive colors that are provided by phytochemical pigments. Anthocyanins are the most common pigments in plants besides chlorophyll and carotenoids. Anthocyanins are biosynthesized via the flavonoid biosynthetic pathway, which is linked to sugar metabolism through the pentose phosphate pathway (PPP). Aroma, a sensory quality of the fruits, is due to the evolution of several complex mixtures of volatile compounds. A number of biosynthetic pathways are involved in production of aroma compounds (Seymour et al., 1993).

21.4.1 Biosynthesis of sugars

In fruits, starch is the major carbohydrate reserve. It is synthesized from glucose-1-phosphate by the action of AGPase (ADP-glucose pyrophosphorylase) enzyme. Starch-degrading enzymes are found in the chloroplast, which convert starch to sugar with fruit ripening. Starch is transformed to glucose-1-phosphate with the action of several enzymes. The glucose-1-phosphate is mobilized into cytoplasm, where sucrose is synthesized by the enzymes UDP-glucose pyrophosphorylase, sucrose phosphate synthase, and sucrose phosphate phosphatase (Paliyath and Murr, 2006). Sucrose is the major sugar, which accumulates as the fruit starts to ripen. With the advancement of ripening, sucrose is further converted to glucose and fructose by the enzyme invertase. In general, glucose and fructose are predominant sugars in ripe fruits of the most species. However, there are exceptions such as mangoes, which show higher level of sucrose with fruit maturation (Selvaraj and Kumar, 1990).

Sugar and sugar phosphates formed during starch catabolism are metabolized through glycolysis, as shown in Fig. 21.1. After a series of reactions, pyruvate is formed during glycolysis and converted to acetyl-CoA in the presence of pyruvate dehydrogenase. Acetyl-CoA serves as a precursor for synthesis of several organic acids, fatty acids, isoprenoids, volatile esters, and phenylpropanoids (Seymour et al., 1993; Paliyath and Murr, 2006). Many organic acids, including malate, citrate, and succinate, are synthesized through the citric acid cycle that generates NADH (nicotinamide adenine dinucleotide) and FADH (flavin adenine dinucleotide), reducing power used for the biosynthesis of ATP.

Sugar and sugar phosphates are also channeled through the PPP, which increases the levels of pentose sugars during ripening. Pentose phosphate pathway provides carbon skeletons for several secondary plant products such as anthocyanins and volatile compounds (Fig. 21.1). Throughout maturation of peaches, high activities of glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate were found, which accumulated higher contents of flavonoids and anthocyanins in fruits (Konga et al., 2007). Higher levels of phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) were also observed with an increase in G6PDH level (Logemann et al., 2000). Based on previous studies, it is clear that G6PDH stimulates anthocyanin biosynthesis. PPP produces reducing power in the form of nicotinamide adenine dinucleotide phosphate (NADPH) that is required during biosynthesis of flavonoids, anthocyanins, isoprenoids, and amino acids (Fig. 21.1). NADPH also plays an important role in the antioxidant enzyme system (Paliyath and Murr, 2006). Therefore, carbon skeletons shift from glycolysis to PPP and subsequently to secondary products.

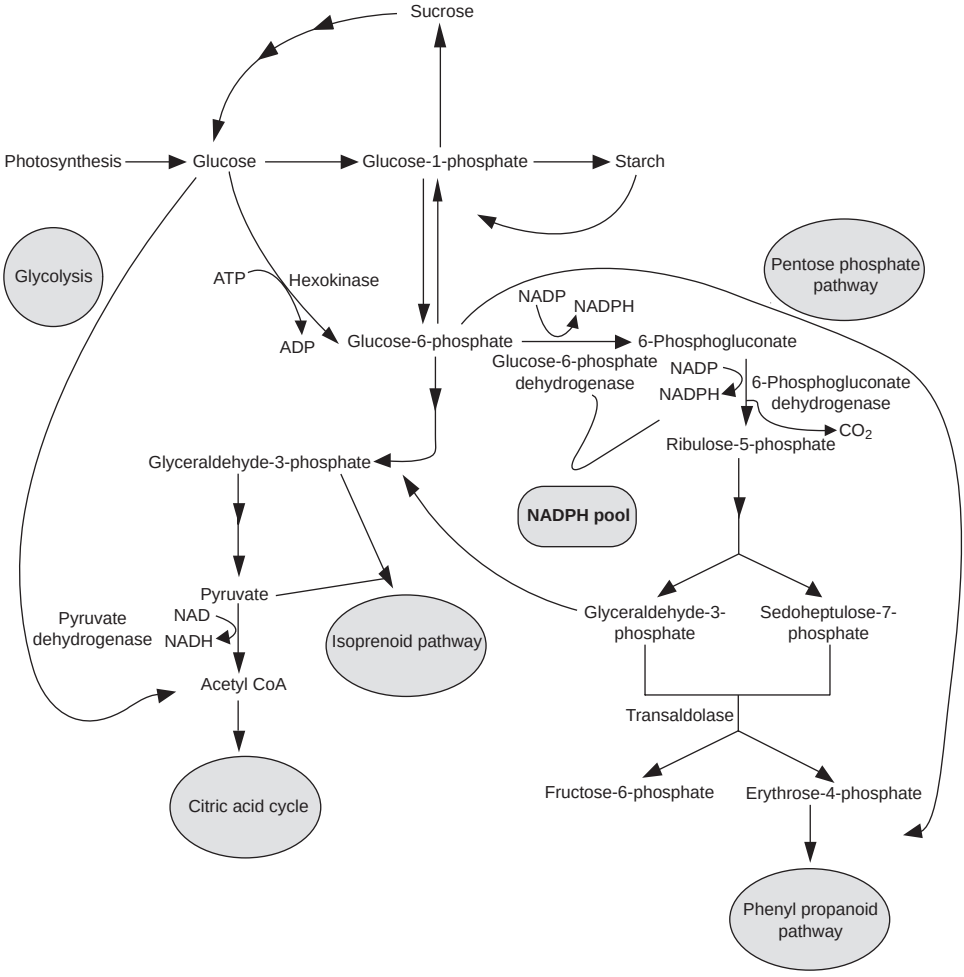

Fig. 21.1 Carbohydrate metabolism in fruits. Starch is catabolized to sugars that undergo glycolysis and pentose phosphate pathway. Pentose phosphate pathway provides carbon and reducing power (NADPH) for the synthesis of flavonoids through phenyl propanoid pathway.

21.4.2 Biosynthesis of isoprenoids

Isoprenoids are secondary plant products that are derived from primary metabolites. Although there are several kinds of secondary plant products, the levels of isoprenoids, ester volatiles, and anthocyanin components determine the fruit quality to a large extent. Isoprenoids are synthesized from two pathways: classical mevalonate (MVA) pathway and 1-deoxyxylulose-5-phosphate (DXP) pathway. These two pathways provide precursors, isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP), for the synthesis of isoprene (Liu et al., 2005b; Paliyath and Murr, 2006). Mevalonate pathway is localized in the cytosol, and DXP pathway occurs in the chloroplast. In mevalonate pathway, three acetyl-CoA molecules undergo condensation to produce 3-hydroxy-3-methyl-glutaryl CoA (HMG-CoA), which forms mevalonic acid (MVA) in the presence of the enzyme

HMG-CoA reductase (HMGR). MVA undergoes phosphorylation, and produces IPP, a five-carbon condensational unit of many terpenes. DMAPP is an isomer of IPP, which is generated in the presence of the enzyme IPP isomerase. These components undergo condensation and produce geranyl (C_{10}), farnesyl (C_{15}), and geranylgeranyl (C_{20}) pyrophosphate compounds, which form monoterpenes, sesquiterpenes, and diterpenes. Monoterpenes and their derivatives (aldehydes, esters, and alcohols) are major volatile compounds in fruits (Paliyath et al., 1997; Paliyath and Murr, 2006). The DXP pathway starts with the condensation of pyruvate and glyceraldehyde-3-P, which generates DXP. Further, a series of reactions produce IPP and DMAPP simultaneously in this pathway (Rohmer et al., 1993).

21.4.3 Biosynthesis of ester volatiles

Every fruit has its own characteristic aroma, which is due to the presence of unique monoterpenes, esters, organic acids, aldehydes, and ketones. During lipid catabolism, several alcohols and acids are generated that combine with fatty acyl CoA to form ester volatiles. Fruits, including banana, apples, and strawberry, are enriched in ester volatiles. With the advancement of ripening, production of volatile components increases. The key enzyme in the synthesis of ester volatiles is alcohol acyl CoA transferase (AAT) that transfers an acyl moiety of the acyl-CoA to a corresponding alcohol (Paliyath and Murr, 2006).

In fruits such as apple, tomato and melon, the aroma formation depends on ethylene production (Bauchot et al., 1998; Fan et al., 1998; Griffiths et al., 1999). It has been reported that repression of ACC synthase and ACC oxidase reduces aroma components in tomato and melon (El-Sharkawy et al., 2005; Zhu et al., 2005). Lipoxygenase activity resulting in the production of hexanal and hexanol is also affected by ethylene biosynthesis in tomato and apple fruits. It is also noted that the use of AVG, an inhibitor of ethylene synthesis, and diazocyclopentadiene, an ethylene action inhibitor, also reduces the biosynthesis of aroma components in apples (Fan et al., 1998). The application of 1-MCP, an ethylene action inhibitor, also decreases aroma volatile production in apples (Fan et al., 1999).

The activity of AAT enzyme was lower in transgenic apples with suppressed ethylene biosynthesis. Furthermore, the exposure of transgenic fruits to 80 μL/L ethylene increased AAT activity to the same level observed in nontransformed apples (Defilippi et al., 2005), suggesting that biosynthesis of flavor volatiles depends on ethylene production. However, it is an open question how ethylene affects the activities of volatile components.

21.4.4 Biosynthesis of anthocyanins

Anthocyanins give red, purple, and blue colors to many fruits, vegetables, cereal, and flowers. The stability of anthocyanins depends on the pH and the chelation of metal ions. They are stable under acidic conditions and are rapidly broken down under neutral conditions. Anthocyanins are glycosylated anthocyanidins, and sugars are mostly attached to the 3-hydroxyl position of the anthocyanidins (sometimes to the 5 or 7 position). There are several hundred types of anthocyanins depending on the number of hydroxyl groups, their methylation patterns, and the nature and number of sugars that are attached the molecule. In higher plants, six anthocyanidins are common that include cyanidin (Cy), delphinidin (Dp), peonidin (Pn), petunidin (Pt), malvidin (Mv), and pelargonidin (Pg).

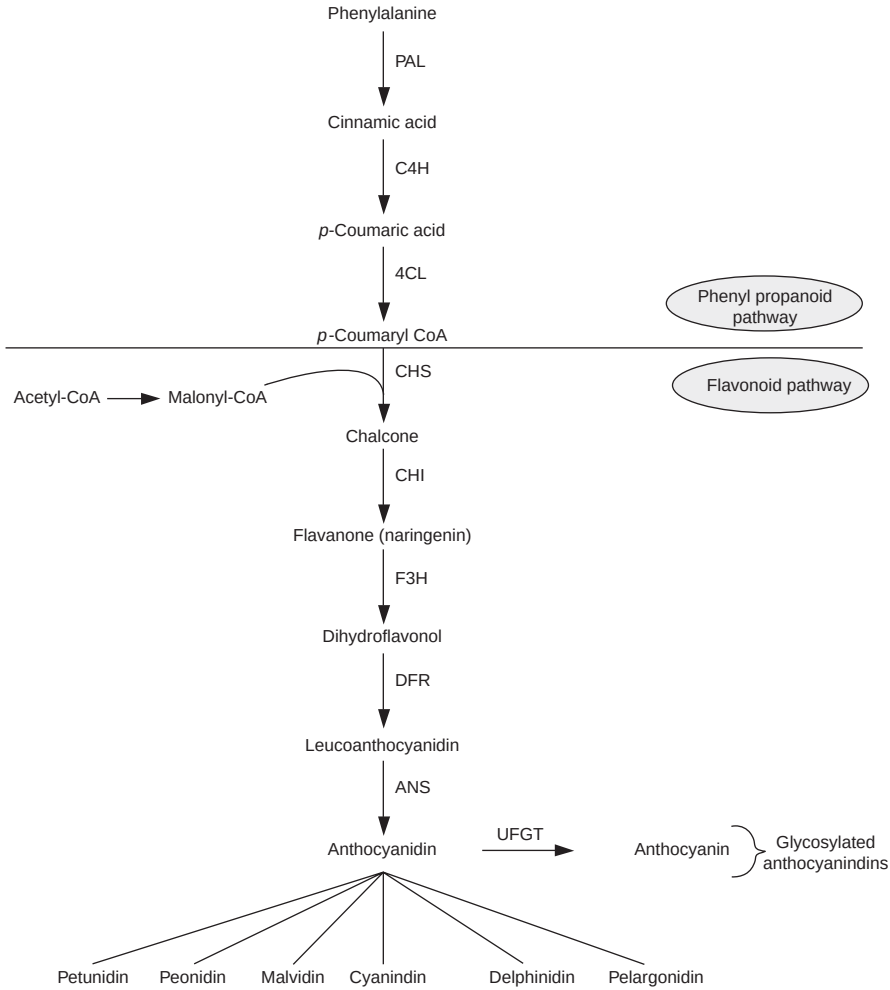

Fig. 21.2 A simplified presentation of anthocyanin biosynthesis in plants. PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hyroxylase; 4CL, 4-coumaryl CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone-3-hydroxylase; DFR, dihydroflavonol-4-reductase; ANS, anthocyanin synthase; UFGT, UDP-glucose flavonoid 3-oxy-glucosyltransferase. (Adapted from Jaakola et al., 2002.)

Anthocyanins are synthesized through flavonoid biosynthetic pathway, which is shown in Fig. 21.2. The first step of anthocyanin biosynthesis is the condensation of three molecules of malonyl-CoA with *p*-coumaroyl-CoA in the presence of the enzyme CHS, which produces chalcone. In the presence of chalcone isomerase, chalcone is converted to flavanone (naringenin) (Jaakola et al., 2002). Flavanone is hydroxylated through flavanone-3-hydroxylase (F3H) and forms dihydroflavonols, which differ in the number of hydroxyl groups. Dihydroflavonol-4-reductase (DFR) transforms dihydroflavonols to colorless leucoanthocyanidin, which yields colored anthocyanidins in the presence of the enzyme anthocyanin synthase (ANS). The glycosylation of anthocyanidins leads to the formation of anthocyanins by the enzyme UDP-glucose flavonoid 3-oxy-glucosyltransferase (UFGT).

Different anthocyanidins give different colors to fruits (Jaakola et al., 2002; Paliyath and Murr, 2006).

In red and nonred apples, differential expression of anthocyanin biosynthetic genes was observed. The expression of *CHS*, *F3H*, *DFR*, and *ANS* genes was found in red apples when anthocyanin was not detected (before ripening stages). However, UFGT gene expression was observed only during the ripening stages with the formation of anthocyanins. This suggests that UFGT gene expression regulates the production of anthocyanins during ripening in apples (Kondo and Hiraoka, 2002). Environmental factors such as light and temperature affect anthocyanin accumulation in fruits. No anthocyanin was found in apples in the absence of light (Proctor, 1974).

21.4.5 Interrelationships of metabolic pathways

In fruits, quality-attributing pathways are interrelated. During starch degradation, glucose-1-phosphate is generated, which enters into several metabolic pathways such as glycolysis and PPP. Glycolysis converts glucose to acetyl CoA after a series of reactions. The acetyl CoA is a precursor for the synthesis of fatty acids, volatile esters, isoprenoids, and organic acids as show in Fig. 21.3. The PPP is an important pathway, which provides carbon skeletons to several biosynthetic pathways including amino acids, secondary metabolites, and nucleic acids. Besides providing carbon skeleton, it also gives reducing energy (NADPH) to various pathways (Fig. 21.3). The carbon skeletons for the synthesis of anthocyanins come from *p*-coumaroyl-CoA and malonyl-CoA. The phenylpropanoid pathway donates *p*-coumaroyl-CoA, which derives from erythrose-4-phosphate and pyruvate that are generated during PPP. Therefore, PPP plays a significant role in the fruit quality regulation.

In flavonoid biosynthesis, the hydroxylation of flavonoids by F3H requires NADPH, which comes from PPP. Isoprenoid synthesis also needs NADPH from PPP (Fig. 21.3). Moreover, it is an important component for the antioxidant enzyme system. The enzymes of ascorbate–glutathione cycle, glutathione reductase (GR), and monodehydroascorbate reductase (MDHAR), require NADPH to inactivate reactive oxygen species (ROS) generated during stress and senescence (Fig. 21.3). In order to maintain fruit quality and shelf life, the precise functioning of the antioxidant enzyme system is necessary.

During membrane lipid catabolism, several fatty acid intermediates are formed that are required for the synthesis of aroma volatile components. The precursors of volatile esters, including hexanal, hexanol, and short-chain alcohols, are also derived from metabolism of lipids. The effect of hexanal has been investigated on PLD activity of membrane, and soluble fractions isolated from corn kernel (Paliyath et al., 1999). Inclusion of hexanal in assay mixture resulted in over 75% inhibition of PLD activity. Other technologies have also been used for the inhibition of PLD activity. A naturally occurring lipid, lysophosphatidylethanolamine (LPE), showed potent inhibition of PLD activity in leaves, flowers, and postharvest fruits. Ryu et al. (1997) noticed a reduction in PLD activity by increasing the length and unsaturation of LPE acyl chain. In addition, decreased ethylene production was found in LPE-treated fruits. Antisense PLD tomatoes showed PLD inhibition. Furthermore, they also possessed higher levels of lycopene, firmness, and soluble solids in the fruits (Pinhero et al., 2003), suggesting that by inhibiting certain metabolic pathways, quality components and shelf life of fruits can be increased.

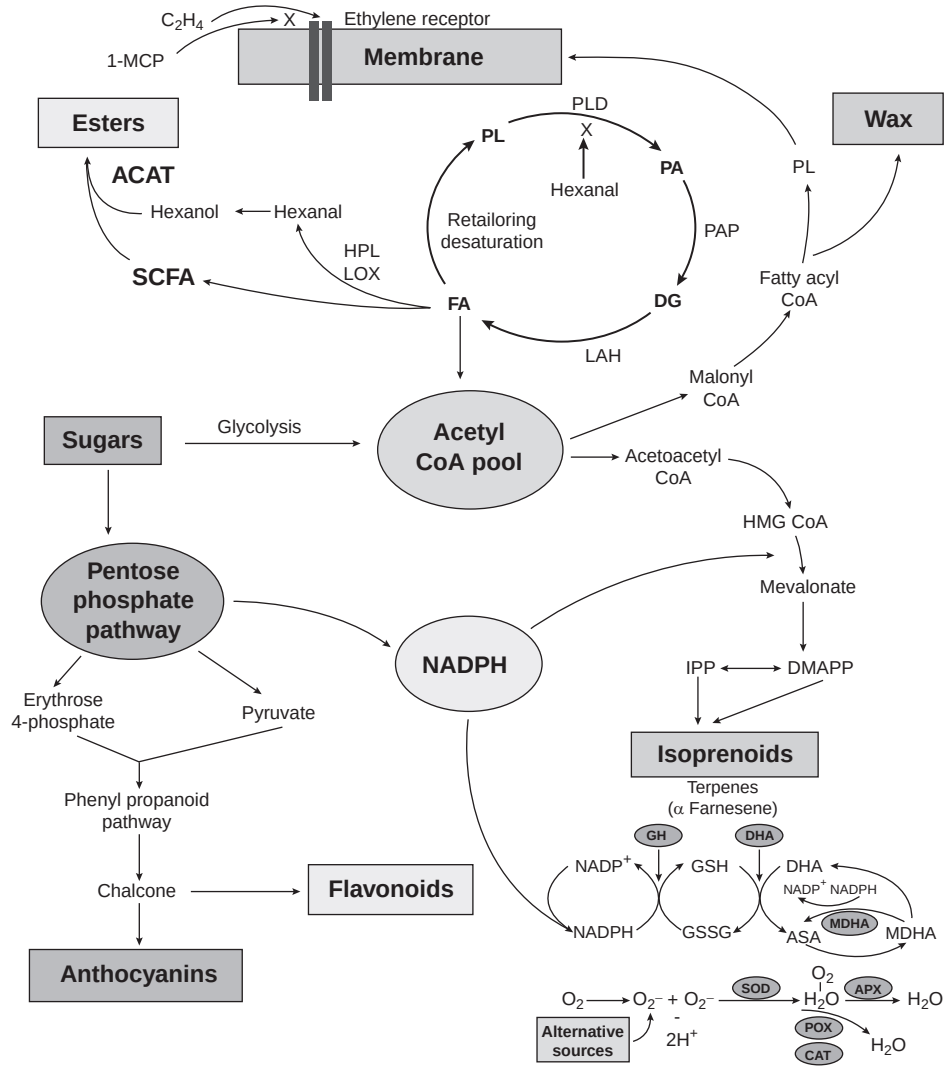

Fig. 21.3 A schematic representation of interrelated pathways and targeted inhibition of catabolic pathways. ACAT, alcohol acyl CoA acyl transferase; APX, ascorbate peroxidase; ASA/MDHA/DHA, ascorbate/monodehydroascorbate/dehydroascorbate; CAT, catalase; DG, diacylglycerol; DMAPP, dimethylallyl pyrophosphate; FA, fatty acids; GR, glutathione reductase; GSH/GSSG, reduced/oxidized glutathione; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; HPL, hydroperoxide lyase; IPP, isopentenyl pyrophosphate; LAH, lipolytic acyl hydrolase; LOX, lipoxygenase; MDHAR/DHAR, monodehydroascorbate reductase/dehydroascorbate reductase; PA, phosphatidic acid; PAP, phosphatidate phosphatase; PL, phospholipid; PLD, phospholipase D; POX, peroxidase; SCFA, short-chain fatty acids; SOD, superoxide dismutase.

21.5 Nutritional components in fruits and their changes during storage

Fruits and vegetables are major sources of vitamins, minerals, polyphenols, antioxidants, and dietary fibers. Their nutritional and health beneficial properties are increasing their demand among consumers. Innumerable studies suggest that a diet rich in fruits and vegetables

reduces the risk of developing cancer, cardiovascular, and several other diseases (Kang et al., 2003; Zhao et al., 2004; Scalbert et al., 2005). Polyphenols have antioxidant properties protecting cells from damaging effects of ROS that are produced during metabolic reactions. The antioxidant and free radical-scavenging properties of polyphenols depend on their molecular structures (such as the position of hydroxyl groups and other features). An imbalance between antioxidants and ROS results in oxidative stress, which leads to the development of cancer, aging, atherosclerosis, cardiovascular disease, and inflammation (Byers and Perry, 1992).

Oxidative damage in cells due to the generation of ROS can be prevented by enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), and glutathione reductase (GR). These damages can also be prevented by antioxidant compounds such as ascorbic acid, α-tocopherols, and carotenoids.

Fruits undergo several changes during harvesting, transportation, and postharvest storage, which affect the nutritional compounds and enzymes involved in the metabolism of those compounds. The changes during prolonged storage periods are related to the taste, nutritional quality, and shelf life of the product. Since every commodity shows different response through storage, it is difficult to preserve nutritional quality of all fruits by a single technology. Thus, it is extremely important to develop sustainable technologies to maintain the quality and shelf life of fruits.

21.5.1 Changes in phenolics and anthocyanins

Polyphenols not only give color and taste to fruits and vegetables, but they also contribute health benefits to human. Qualitative and quantitative compositions of polyphenols differ from one fruit to another. Even within the same species, a large variation in polyphenols can be found in the cultivars. For example, in sweet cherry, cyanidin-3-rutinoside is the predominant anthocyanin. Sweet cherries "Bing" contain high level (180 mg/100 g flesh weight) of cyanidin-3-rutinoside, whereas "Summit" contains low levels (72 mg/100 g flesh weight) of this anthocyanin (Gao and Mazza, 1995). However, in case of sour cherries, cyanidin-3-glucosylrutinoside is a major anthocyanin. A higher level of cyanidin-3-glucosylrutinoside (227 mg/100 g fresh weight) was found in "Sumadinka" sour cherry, while "Balaton" had only 88 mg/100 g fresh weigh cyanidin-3-glucosylrutinoside (Kim et al., 2005).

Plant phenolics are highly unstable and they undergo various changes throughout storage. These changes are associated with taste and nutritional quality of fruits. Fruits like cherries, strawberries, and litchi are highly perishable, and they start to develop brown pigments within 2–3 days of harvest, at ambient temperature. Postharvest browning of fruits is mainly due to the breakdown of anthocyanins and oxidation of phenolics. In the presence of oxygen, phenols are oxidized by polyphenols oxidase (PPO), which catalyzes two reactions: (i) the hydroxylation of monophenols to *o*-diphenols and (ii) the oxidation of *o*-diphenols to quinones, slightly colored compounds, condensed to form brown pigments (melanins) (Macheix et al., 1990). The free phenolic compounds are predominately localized in the vacuole, and PPO is localized in chloroplast and cytoplasm. During storage, subcellular decompartmentalization leads to the enzyme and substrate coming into contact, which triggers browning in fruits and vegetables (Macheix et al., 1990; Tomas-Barberan and Robins, 1997). Several factors, such as nature and substrate content, enzyme activity,

oxygen availability, and enzyme-substrate contact, also determine the extent of browning in fruits.

POX may also contribute to the enzymatic browning in fruits (Nicolas et al., 1994). These enzymes are involved in oxidation of phenolics in the presence of hydrogen peroxide. POX can rapidly oxidize 4-methylcatechol in the presence of H_2O_2. In pears, PPO can elevate POX activity by generating H_2O_2 during oxidation of phenols. POX further oxidizes phenols by using quinones as a substrate (Richard-Forget and Gauillard, 1997). Thus, POX activity depends on PPO for its role in enzymatic browning. Zhang et al. (2005) reported that during storage of litchi, POX activity was increased, which enhanced enzymatic browning in the fruit pericarp. In addition, anthocyanin concentration decreased with the enhancement of browning and POX activity in litchi. This indicates that POX plays an important role in anthocyanin degradation with pericarp browning in litchi pericarp (Zhang et al., 2005).

Postharvest handling and transportation induce wounding, resulting in cell disruption and loss of compartmentalization. This damage can stimulate the leakage of phenolics from vacuoles enabling the contact of enzymes with their substrates. Higher POX activity was found in damaged mangosteen fruits with decreased phenolic content and enhanced lignin synthesis in the presence of oxygen (Ketsa and Atantee, 1998). However, a significant increase in the activity of PAL, the first committed enzyme in the biosynthesis of phenolics, was found during wounding in lettuce that subsequently increased phenolic content (Saltveit, 2000). Higher phenolic contents in lettuce enhanced PPO and POX activity, which induced browning.

21.5.2 Storage temperature and phenolic compounds

Storage at low temperature is a good method to reduce the activities of PPO and POX enzymes. However, depending on the commodity and storage temperature, cold storage has positive and negative effects on phenolic compounds. PPO and POX activities vary from one fruit to another and even within different cultivars of the same species. It has been reported that low temperature (5°C) and normal atmospheric storage of apricots for 10 days decreased the browning rate and increased the POX (Vámos-Vigyázó et al., 1985). However, there was an irregular change in PPO activity. This suggests that there are other factors besides enzyme activity and substrate concentration that influence browning in fruits.

Phenolic contents of fruits are highly influenced by the degree of ripeness, cultivars, storage conditions, and environmental factors. Dissimilar changes in the pattern of individual phenolic content have been observed in different fruits. In general, an increase in anthocyanins has been found during storage of fruits at low temperature. Fruits such as strawberries (Gil et al., 1997; Sanz et al., 1999), blueberries (Kalt and McDonald, 1996), and pomegranate (Holcroft et al., 1998) have shown high anthocyanin contents at low-temperature storage. Gonçalves et al. (2004) reported that the total phenolic content increased in sweet cherry during storage at both 1–2°C and 15 ± 5°C. However, the levels of total phenolics were higher in cherries kept at room temperature (15 ± 5°C) than cold storage (1–2°C). They also found a variation in the levels of individual phenolics, but this variation was lower in cold storage than room temperature (Gonçalves et al., 2004).

In Canada, the marketing season for fruits such as sweet cherry lasts from mid-June to August, which is rather short. Due to the short marketing season, it is consumed in several forms including frozen, canned, and juice. Recently, it has been found that anthocyanin

and phenolic contents of cherries decreased during storage at −23 and −70°C. In frozen Bing cherries, ∼75% anthocyanin content was lost when stored at −23°C during storage for 6 months (Chaovanalikit and Wrolstad, 2004). A reduction in total phenolic content was also found in frozen cherries, but it was not as high as in anthocyanins. The severe reduction in anthocyanin content could be due to the presence of PPO activity. This enzyme plays an important role in deterioration of quality of the most fruits and vegetables. At freezing temperature, solute concentration is increased that helps in catalysis of chemical reactions. Moreover, freezing increases membrane decompartmentalization and induces enzyme-substrate contact (Cano et al., 1995). Similar patterns were also noticed in frozen papaya (Cano et al., 1995). Therefore, long-term storage of fruits at freezing temperature decreases the nutritional quality of the fruits.

Storage of some fruits at low temperature induces chilling injury characterized by reduced fruit quality, increased browning, and off-flavor development in fruits. Different fruits show chilling injury at different temperatures. In general, fruit skin is more sensitive to develop chilling injury than fruit flesh. It has been observed that the chilling injury is positively correlated with the activities of PAL (Martínez-Téllez and Lafuente, 1997; Lafuente et al., 2001) and PPO enzymes (Nguyen et al., 2003). However, no association between chilling injury and changes in PPO and POX has been found in some mandarin (Martínez-Téllez and Lafuente, 1997) and orange (Martínez-Téllez and Lafuente, 1993) cultivars. It is found that chilling also increases ethylene production. Exogenous or endogenous ethylene produced during stress conditions may provoke PAL activation (Lafuente et al., 2001). An inverse relationship between free phenolics and chilling injury was also noticed (Nguyen et al., 2003). This relationship may be because of membrane damage during chilling. In a recent report, it has been reported that the antioxidant activity and phenolics levels remained relatively stable in several fruits during room temperature storage or refrigerated storage at 4°C. In some cases, an increase in anthocyanin levels was also observed (Kevers et al., 2007).

21.5.3 Controlled atmosphere storage and phenolic compounds

Since oxygen plays a pivotal role in oxidation of phenolics at normal atmosphere, the complete removal of oxygen may control the phenolic oxidation. However, due to the risk of anaerobic respiration, it is not a practical approach. The storage of fruits under CA with reduced O_2 and increased CO_2 can be a favorable method in order to preserve the quality of fruits. Controlled atmosphere in conjunction with low temperature can be a crucial factor to regulate respiratory and metabolic activities of perishable commodities.

Several studies have evaluated the effect of CA on fruits and vegetables. It has been observed that browning in fruits and vegetables was decreased by increasing the CO_2 level in storage atmosphere (Buescher and Henderson, 1977; Siriphanick and Kader, 1985). CA storage of "Delicious" apples for 7–14 weeks showed a major reduction in PPO activity and browning (Barrett et al., 1991). During CA storage, lower PPO activity was also noticed in minimally processed apple slices. All CA-stored apples exhibited lower PPO activity than air-stored ones. Furthermore, it has been observed that the higher CO_2 concentrations had the maximum effect on PPO activity inhibition (5–30%) (Awad and Jager, 2003). At high CO_2 concentrations, higher phenolic content and lower browning index were also found, probably, due to lower PPO activity. In several fruits, the CO_2 levels above 5% inhibited PPO activity and finally reduced browning.

Gil et al. (1997) indicated that the treatments with CO_2 had direct effect on strawberry anthocyanin levels. Fruits stored in air showed higher anthocyanin levels than CO_2-treated fruits at 5°C. A rapid decline in the anthocyanin levels was noticed with higher concentrations of CO_2. Fruits stored at higher CO_2 atmosphere showed a prominent decrease in the anthocyanin content of internal tissues, whereas fruits stored in air did not show any effect on anthocyanin levels in both internal and external fruit tissues. An increase in the pH of internal tissues was also noticed with an increase in CO_2 concentrations, which may be the cause of anthocyanin degradation (Gil et al., 1997). The decrease in anthocyanin concentration at high CO_2 atmosphere (12% CO_2) was also monitored in sweet cherry; however, high atmospheric CO_2 reduced the activities of PPO and POX during postharvest storage of sweet cherries (Remòn et al., 2004). On the other hand, no changes in total phenolics and flavonoids were found during CA storage of cranberry fruits (2, 21, and 70% O_2 with 0, 15, and 30% CO_2) at 3°C (Gunes et al., 2002). Based on these studies, it can be concluded that CA can have pronounced effects on metabolism of phenolics depending on the commodity.

Modified atmosphere packaging (MAP) is also used to reduce the oxygen level around the products. Polyvinyl chloride, polyethylene terephthalate, polyethylene, and polypropylene are the major films used in MAP. The main gases used in MAP are oxygen, nitrogen, and carbon dioxide. Depending on the product sensitivity and color stability to these gases, different proportions of gases are used in MAP.

MAP influences browning and PPO activity. A significant depletion in PPO activity was observed in minimally processed "Golden Delicious" apples under MAP (90.5% N_2 + 7% CO_2 + 2.5% O_2 and plastic pouches) (Soliva-Fortuny et al., 2001). However, no change in color was found in ready-to-eat apples during the storage period. The selection of atmospheric composition and packaging material significantly affects the PPO activity and color loss in the products, and maintains the shelf life and nutritional quality of the products. Broccoli wrapped in low-density polyethylene film lost its nutritional content during refrigerated transportation; however, when packed with microperforated (Mi-P) or nonperforated (No-P) films, functional compounds such as polyphenols were preserved during storage (Serrano et al., 2006). Therefore, in general, MAP is a beneficial method to maintain the nutritional quality and extend the shelf life of fruits and vegetables.

21.5.4 Growth regulator treatments and phenolic compounds

Fruits and vegetables are exposed to plant hormones and other chemicals to extend their postharvest life and to maintain their nutritional contents. Several chemicals, including 1-MCP, hexanal, methyl jasmonate (MJ), and abscisic acid (ABA), have shown their effects on browning, phenolic compounds, and PPO and PAL activities. Loquat fruits exposed to 1-MCP for 12 h showed lower PPO activity and browning index during storage (Cai et al., 2006). During storage period, an increase in total phenolics was also noticed with the decline in PPO activity in 1-MCP-treated fruits. 1-MCP-treated apples also exhibited higher flavonoid contents as compared to control apples (MacLean et al., 2006). However, in sweet cherry, 1-MCP treatment failed to intensify total anthocyanins and hydroxycinnamic acids, the major polyphenols in cherries, during cold storage (Mozetič et al., 2006).

The effect of 1-MCP also depends on harvest maturity. Optimum maturity of the product is an important factor to get the desirable effects from these postharvest treatments. 1-MCP treatment in early-harvested apples showed less chlorogenic acid than optimal matured and

late-harvested apples (MacLean et al., 2006). Therefore, plant developmental stage must be considered to achieve maximum effect from the treatment.

Naturally occurring compounds such as hexanal, released from lipoxygenase pathway, may prevent browning in fruits. Apple slices treated with hexanal have shown fruit color stability and reduction in browning and PPO activity during storage (Lanciotti et al., 1999; Corbo et al., 2000). In tissues, hexanal is converted to hexanol (aliphatic alcohol) that would help in the prevention of browning by reducing PPO activity. Besides providing color stability, this natural compound, hexanal, has antimicrobial activity (Corbo et al., 2000). It has been noticed that the effect of hexanal on color retention was improved by increasing the storage temperature. Nevertheless, hexanal treatment in table grapes did not show any significant effect on phenolic content (Artés-Hernández et al., 2003).

One of the most effective PPO inhibitor is sulfur dioxide (SO_2), which is widely used in food industry. However, due to its negative effects on health, the need for alternatives increased. Compounds, such as ABA, and formulation of ascorbic and citric acids, have proved their potential role as antibrowning agents (Rocha and Morais, 2005). ABA has been reported to enhance anthocyanin accumulation and PAL activity. Exogenous application of ABA on cherry and strawberry promoted anthocyanin synthesis in the fruits (Hartmann, 1992; Jiang and Joyce, 2003). In apples, a dip treatment in 1% ascorbic acid and 0.2% citric acid resulted in 90–100% inhibition in PPO activity (Rocha and Morais, 2005). Reduction in POX activity was also noticed in ascorbic acid–treated cantaloupe melons (Lamikanra and Watson, 2001). It could be due to the antioxidant property of ascorbic acid, which may reduce oxidative stress in fruit.

Methyl jasmonate derived from lipoxygenase-dependent oxidation of fatty acids is a naturally occurring volatile compound in plants that is involved in plant growth, development, and stress conditions. Preharvest treatment with MJ significantly increased the anthocyanins and total phenolics in raspberry (Wang and Zheng, 2005). MJ-treated fruits also showed stimulation in ethylene synthesis, which might provoke enzymes such as PAL. Exogenous application of MJ also induces defense compounds, such as polyphenols, preventing fruits from fungal attack during storage (Wang and Zheng, 2005). The combined treatment of MJ and ethanol also enhanced phenolic and anthocyanin contents in strawberry during storage period (Ayala-Zavala et al., 2005).

21.5.5 Changes in antioxidant activity

Metabolism of fruits continues even after detachment from the plant. During storage, fruits and vegetables undergo several stress conditions including wounding, chilling, heat, pathogens, and senescence. These changes generate reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), hydroxyl ($\cdot OH$), and peroxyl radicals and superoxide anion ($O_2^{-\cdot}$), which result in cellular deterioration including lipid peroxidation, enzyme inactivation, and mutation (Halliwell, 2006). Plant cells are protected or alleviated from ROS by an antioxidant system that works as ROS scavenger. In plants, two types of antioxidants are found: enzymatic antioxidants and nonenzymatic antioxidants. Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), and glutathione reducatse (GR), and nonenzymatic antioxidants include flavonoids, ascorbic acid, α-tocopherol, and β-carotene (Blokhina et al., 2003).

Antioxidant enzymes remove ROS before their participation in oxidation reactions, which subsequently prevent initiation of oxidation. In the plant cells, the first line of defense enzyme is SOD converting the superoxide radical ($O_2^{-\cdot}$) to H_2O_2. Since excessive amount of H_2O_2 is toxic to the cells, it is further decomposed by CAT and POX, which convert H_2O_2 to water. By using two molecules of ascorbate, APX reduces H_2O_2, and generates monodehydroascorbate (MDHA) and dehydroascorbate (DHA), oxidized forms of ascorbate (Larrigaudière et al., 2004). However, during postharvest handling and storage, fruits sustain stress, such as senescence and superficial scald, which alter the levels of antioxidant constituents. In general, the levels of antioxidants increase during moderate stress conditions, while, with the advancement in stress, a decline in antioxidant constituents has been observed, which results in the development of postharvest disorders (Barden and Bramlage, 1994; Purvis et al., 1995; Shewfelt and Purvis, 1995). Various postharvest factors, such as duration and temperature of storage, can influence stress in fruits and vegetables and eventually alter the levels of antioxidants.

21.5.6 Harvest maturity and antioxidants

Harvest maturity is also a critical factor for antioxidant constituents. An enhancement in the lipid-soluble antioxidants was found in apples during storage, which was related to their levels at harvest (Barden and Bramlage, 1994). In addition, scald development during storage was negatively correlated with the concentration of antioxidants at the time of harvest. Tomato fruits ripened off the vine showed higher levels of antioxidants (lycopene, β-carotene, phenolic, and ascorbic acid) during storage than vine-ripened fruits (Giovanelli et al., 1999).

Early-harvested "Braeburn" apples showed higher SOD activity, which reduced in the later-harvested fruits (Gong et al., 2001). However, Golden Smoothee apples showed higher total antioxidant activities (SOD, CAT, and POX) in late-harvested fruits (Molina et al., 2005). This indicates that nutritional components in fruits are affected by harvest maturity, which may vary from one fruit to another. Therefore, optimal harvest date should be determined for individual fruit.

21.5.7 Storage temperature and antioxidants

Generally, changes in environmental conditions result in changes in antioxidant metabolism. Low-temperature storage decreases metabolism in fruits and vegetables and minimizes the risk of damage during storage. Delay between harvesting and cooling can decline the nutritional quality of the products. A 10% reduction in ascorbic acid content was found in leafy vegetables stored at 6°C for 6 days. In contrast, storage at room temperature for only 2 days resulted in a 20% reduction in ascorbic acid content (Lee and Kader, 2000). Similarly, lycopene content in tomatoes kept at 7°C was lower than tomatoes kept at 15 and 25°C. Ascorbic acid content was found to be stable in tomatoes during storage potentially due to the high acidity of the fruit (Toor and Savage, 2006).

Kiwifruit slices stored at 0, 5, and 10°C for 6 days showed 8, 12.8, and 20.6% reduction in total vitamin C content, respectively (Agar et al., 1999). Since ascorbic acid is the predominant form of vitamin C, the reduction was higher in ascorbic acid content. A decline in ascorbate content was found in raspberries and low-bush blueberries during storage from

0 to 10, 20, and 30°C (Kalt et al., 1999). Storage temperatures did not show any effect on the ascorbate content in strawberry and high-bush blueberry. Connor et al. noticed an increase in antioxidant activity in blueberry during cold storage (Connor et al., 2002). Higher antioxidant activity was directly related to the anthocyanin and phenolic contents of blueberry during storage. In contrast, no difference in antioxidant capacity was found in fresh, frozen, and cold-stored raspberries (Mullen et al., 2002). Sweet cherry storage at −23°C for 6 months exhibited 58% decline in antioxidant activity, while fruits stored at −70°C for 6 months showed 45% increase in antioxidant activity (Chaovanalikit and Wrolstad, 2004).

An increase in the level of GR was observed in apples during storage for 1–3 months at 2°C. However, no change in ascorbate POX activity was noticed during storage (Barbara and Marzenna, 2002). It has been indicated that CAT activity was reduced in apples during freezing. Frozen apples showed 70–80% lower CAT activity than fresh apples (Gong et al., 2000). Apple tissues kept at −25°C showed higher loss in CAT activity than tissues kept at −70°C. It appears that if the freezing process is slow, the degradation in CAT activity is higher. The freezing process did not show any significant effect on activities of other enzymes including SOD and POX (Gong et al., 2000).

Low-temperature storage shows chilling injury in chilling-sensitive horticultural crops. Cucumber stored at 5°C showed low ascorbic acid content, while no reduction in ascorbic acid was noticed at 20°C (Lee and Kader, 2000). Low-temperature storage of chilling-sensitive mandarins increased chilling injury of fruits and decreased antioxidant enzyme (POX, APX, and CAT) activities. However, chilling-tolerant fruits exhibited lower chilling injury and higher antioxidant enzyme activities. It indicates that chilling-tolerant cultivars have more efficient antioxidant system than chilling-sensitive cultivars (Sala, 1998).

21.5.8 Controlled atmosphere storage and antioxidants

Controlled atmosphere, with elevated CO_2 concentrations, is widely used to maintain the shelf life and nutritional quality of several fruits and vegetables. In general, high CO_2 concentrations have shown a reduction in ascorbic acid content in the products. During storage in CA with high CO_2 concentrations (20 and 30%), strawberries displayed a decrease in ascorbic acid and vitamin C contents, while air-stored strawberries did not show any reduction in the ascorbic acid and vitamin C contents (Agar et al., 1997). Similar patterns were also found in red currants and blackberries. The strawberries with reduced ascorbic acid showed an increase in DHA level, which might be due to the stimulation of ascorbic acid oxidation by APX in the presence of high CO_2. Moreover, it is believed that high CO_2 may inhibit MDHAR and (dehydroascorbate reductase) DHAR activities (Agar et al., 1997). Apples kept at a high CO_2 atmosphere also showed a loss in ascorbic acid content. A reduction in vitamin C content in kiwifruit slices has been noticed with increasing CO_2 concentrations. Slices kept in air + 5, 10, or 20 kPa CO_2 accelerated the loss of vitamin C by 14, 22, or 34%, respectively (Lee and Kader, 2000).

The antioxidant activities of air-stored cranberries increased by 50% from their harvest levels in 2 months, while CA-stored fruits (21% O_2 + 30% CO_2) prevented this increase (Gunes et al., 2002). This may result from an impediment in the release of bound phytochemicals during the CA storage, which contribute to antioxidant activity. However, one study reported that apples stored in cold or CA did not show any effect of storage on

antioxidant activity (Sluis et al., 2001). An increase in antioxidant activity during storage has been observed in apples stored in CA (2% CO_2 + 2% O_2) or cold storage for 4 months (Leja et al., 2003).

Off-flavor compounds such as ethanol and acetaldehyde start to accumulate during long-term storage of pears in high CO_2 atmosphere that promotes progression of physiological disorders including core browning (CB). It has been noticed that pears stored in CA with high CO_2 concentrations developed rapid injury. In CA-stored pears, H_2O_2 accumulated rapidly indicating that fruits undergo stress from changes in O_2 and CO_2 concentrations. Short-term storage of "Conference" pears under CA (2% O_2 and 5% CO_2) exhibited an increase in SOD and APX activities and a decrease in CAT activity (Larrigaudière et al., 2001). Higher lipoxygenase enzyme activities were also reported in CA-stored pears, which induced membrane lipid peroxidation.

Broccoli has health-promoting compounds such as flavonoids, indole-3-carbinol, and vitamin C that provide antioxidant capacity to freshly harvested broccoli. Reports suggest that these antioxidant compounds degrade during storage. Broccoli, packed in MAP using microperforated and nonperforated films, maintained the levels of total antioxidant activity and phenolic content even after 28 days of storage, whereas a rapid decline in these health-promoting compounds was monitored in control broccoli after 20 days of cold storage (Serrano et al., 2006).

A significant decline in total antioxidant activity was observed in ready-to-eat shredded purple carrots stored under MAP (95% O_2 + 5% CO_2), while carrots stored at 90% N_2 + 5% O_2 + 5% CO_2 maintained antioxidant activity for 10 days (Alasalvar et al., 2005). Therefore, CA storage can preserve health-promoting components in some cultivars, while high CO_2 concentration may reduce the ascorbic acid content and antioxidant activity in some fruits including strawberry and pears.

21.5.9 Growth regulator treatments and antioxidants

The application of several pre- and postharvest treatments have been investigated to reduce the oxidative stress and to increase the nutritional value of the products. Antioxidant chemicals as dips or coatings have been used in several studies to prevent the oxidative reactions (Sapers, 1993). An increase in ascorbic acid content was observed in 1% $CaCl_2$ dip–treated kiwifruit slices. A further increase in ascorbic acid content was noticed when 1% $CaCl_2$-treated slices were kept in an ethylene-free atmosphere (Agar et al., 1999). An increase in vitamin C content was also found in $CaCl_2$-treated apples (Lee and Kader, 2000). In a study, 2% $CaCl_2$ treatment of apples maintained higher amount of ascorbic acid content after 60 days of storage than 1.5 and 1% $CaCl_2$ treatment (Hayat et al., 2005). Calcium dips have also shown delayed membrane deterioration and senescence (Lester, 1996; Picchioni et al., 1998). Calcium induces tolerance to tissue injury by enhancing antioxidant activity.

Pre- and postharvest treatments of fruits and vegetables with plant growth regulators and natural volatile compounds have shown positive effects on antioxidant activity. Methyl jasmonate, a natural volatile compound, increased the contents of ascorbate, dehydroascorbate, and phenolics in raspberries during storage compared to control fruits. Moreover, methyl jasmonate–treated raspberries showed higher activities of SOD, POX, APX, MDHAR, and DHAR (Chanjirakul et al., 2006). Methyl jasmonate–treated strawberries and blueberries

also showed the same results (Chanjirakul et al., 2007). This suggests that methyl jasmonate treatment enhances antioxidant activity and free radical-scavenging capacity in fruits.

During inadequate antioxidant activity, free radicals cause membrane deterioration resulting in CB in pears. Previous studies have reported the negative correlation between CB and antioxidant metabolism. A reduction in antioxidant enzymes (SOD, POX, and CAT) has been noticed in CB pears (Fu et al., 2007). 1-MCP treatment has shown higher CAT, POX, and SOD activities in treated fruits compared to the control fruits during storage. Furthermore, the incidence of CB in 1-MCP-treated fruits was 91% lower than in the control fruits. During storage, 1-MCP-treated fruits exhibited a significant reduction in H_2O_2 level, which also indicates an increase in the free radical-scavenging capacity during the storage (Larrigaudière et al., 2004). Therefore, 1-MCP enhances the activities of antioxidant enzymes and reduces the occurrence of CB in pears. So, it eventually reduces the development of physiological disorders during storage.

In apples, scald-susceptible fruits had higher H_2O_2 concentration than scald-resistant fruits during storage, and these lower H_2O_2 concentrations were related to lower scald development. In addition, higher activities of POX and CAT were also associated with lower H_2O_2 levels (Rao et al., 1998). Hence, antioxidants enhance the tolerance to environmental stress. Lower contents of lipid-soluble antioxidants were noticed in scald-affected apples during storage. In contrast, healthy fruits exhibited higher content of lipid-soluble antioxidants. With 1-MCP treatment, the levels of lipid-soluble antioxidants, α-tocopherols, and water-soluble antioxidants, ascorbic acid, phenols, and glutathione, were increased in scald-affected apples. Moreover, 1-MCP treatment subsequently reduced the fruit scald susceptibility during the storage (Shaham et al., 2003). Therefore, postharvest treatments may reduce the storage stress in fruits and increase the levels of antioxidants that play an important role in nutritional quality of the products.

21.6 Changes in sugars

Glucose, fructose, and sucrose are the main sugars in fruits. The right proportion of these sugars attributes to the quality of the fruits. The sweetness of fructose is 1.8 times higher than sucrose, whereas the sweetness of glucose is 3/5 of sucrose (Wang and Zheng, 2005). The time of harvest significantly influences carbohydrate contents in fruits. The respiration rate increases during postharvest storage at ambient temperature. An increase in respiration enhances the consumption of sugars as substrates for several metabolic processes. The first substrate used during respiration is sugar. A close relationship between respiration and sugar levels was noticed in peaches during storage (Chen et al., 2006). There are several methods, such as low temperature and CA storage and postharvest treatments, to delay postharvest changes in the fruits and vegetables

21.6.1 Storage temperature and sugars

Low-temperature storage has been tried to maintain sugar levels in harvested fruits. Papaya slices kept at 20°C had lower total soluble solids (TSSs) than slices kept at 5 or 10°C (Rivera-Lòpez et al., 2005). Depletion of soluble solids at high temperature can be explained by a high respiration rate. During storage of frozen papaya, an increase in glucose and fructose

contents was observed, while the levels of sucrose decreased (Torija et al., 1998). The explanation is due to hydrolysis of the sucrose to glucose and fructose by invertase.

A decline in sucrose content was also observed in pears during storage, while the levels of fructose and glucose increased during 5 months of storage at 0°C (Chen et al., 2006). A similar trend was also observed in apples. The contents of glucose, fructose, and sucrose changed in apples during the storage at low temperature. The levels of glucose, fructose, and sorbitol increased and sucrose decreased during storage at 1°C for 90 days in air (Drake and Eisele, 1999). On the other hand, there was no difference found in TSSs of apples stored in air and CA.

21.6.2 Controlled atmosphere storage and sugars

Sweet cherries are more tolerant of high CO_2 concentration than other temperate fruits. In addition, high-soluble solids containing cherries have a lesser risk of low O_2 concentration damage. "Stella" and "Van" cherries stored at high CO_2 concentrations (>10%) exhibited less decay than cherries stored at lower CO_2 concentrations. In contrast, CO_2 concentration in CA (15% CO_2 + 21% O_2) did not show any significant effect on soluble solids of cherries (Wang and Vestrheim, 2002). Similar results were also found in "Burlat," "Bing," and "Sweetheart" cherries stored in CA and MA (Remòn et al., 2004).

Holcroft and Kader (1999) reported that strawberry storage at high CO_2 concentration (20 kPa CO_2) in CA showed a decline in sucrose, fructose, and glucose at 5°C for 10 days storage period. Nevertheless, these concentrations were higher in 0.5 kPa O_2. Overall, the concentration of sucrose decreased, and the concentrations of glucose and fructose increased during 10 days of storage in 0.5 kPa O_2 atmosphere. However, TSS content was decreased with storage time. This could be due to the fact that TSS measures the levels of sugars, organic acids, and soluble pectins.

21.6.3 Growth regulator treatments and sugars

Natural volatile compounds, including methyl jasmonate and jasmonic acid, have positive effects on sugar content of fruits. Raspberry fruits treated with methyl jasmonate had higher-soluble solids content than the control fruits. Treated fruits contained higher levels of fructose, glucose, and sucrose than the control fruits (Wang and Zheng, 2005). A combined treatment of methyl jasmonate and ethanol also increased the soluble solids content in strawberries (Ayala-Zavala et al., 2005). Similar results were also found in jasmonic acid–treated apples (Wang and Zheng, 2005). Low respiration may be the cause of high sugar contents in treated fruits, while high metabolism in control fruits may justify the depletion of carbohydrate contents.

Postharvest treatment with 1-MCP has been explored to enhance the carbohydrate contents in apples (Perera et al., 2003), peaches (Liu et al., 2005a), nectarines (Bregoli et al., 2005), and pears (Fu et al., 2007). However, none of these studies has shown any significant effect of 1-MCP on fruit sugar levels. This may suggest that ethylene does not affect free sugar levels in fruits. On the other hand, an increase in soluble solids has been noticed during ripening (Perera et al., 2003).

Thus, it is apparent that the postharvest quality of fruits may change during storage depending on several preharvest and postharvest factors. Therefore, it is essential to optimize

potential storage methods for every commodity to obtain a produce having ideal organoleptic quality as well as the best nutritional quality.

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