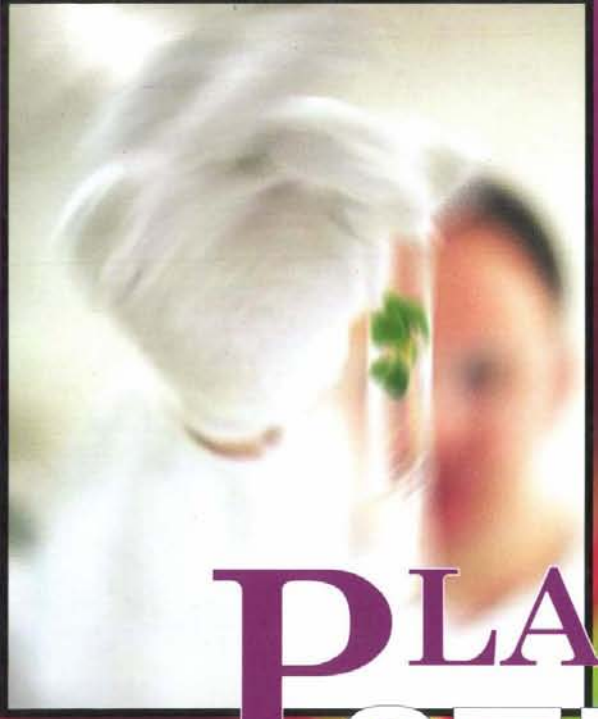

PLANT STRESS AND BIOTECHNOLOGY

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Plant Stress and Biotechnology

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People's Republic of China

Oxford Book Company
Jaipur, India

ISBN : 978-81-89473-10-5

First Published 2007

Oxford Book Company
267, 10-B-Scheme, Opp. Narayan Niwa
Gopalpura By Pass Road, Jaipur-302018
Phone: 0141-2594705, Fax: 0141-2597527
e-mail: oxfordbook@sify.com
website: www.abdpublisher.com

Typeset by :
Shivangi Computers
267, 10-B-Scheme, Opp. Narayan Niwas,
Gopalpura By Pass Road, Jaipur-302018

Printed at :
Rajdhani Printers, Delhi

Contents

1 Homeodomain-Leucine Zipper Proteins Participating in Abiotic Stress Response in Plants

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Introduction

Development in multicellular organisms results from growth and differentiation and is determined by a specific program of gene expression. In plants, environmental factors have a great influence on development via different signal transduction pathways that amplify the original signal and ultimately result in the activation or repression of certain genes. The synthesis of most eukaryotic proteins is regulated at the transcriptional level. Such a coordinated regulation depends on the activity of a group of proteins generally called transcription factors, which are able to enhance or reduce the rate of transcription by facilitating the assembly of the initiation complex. A typical minimum promoter extends about 100 bp upstream of the transcription initiation site and includes several sequence elements named proximal promoter sequences. The promoter sequences that interact with transcription factors are termed *cis*-acting elements while transcription factors that bind these *cis*-acting sequences are called *trans*-acting factors. *Cis*-acting elements that are far before the proximal promoter can exert either positive or negative control and are termed distal regulatory sequences. Distal *cis*-acting elements involved in gene regulation by hormones and other signaling agents are called response elements.

Coming to *trans*-acting elements, it is possible to identify in a typical transcription factor, three structural features: a DNA binding domain, a transcription-activating domain and a ligand-binding domain. The DNA-binding domain must be able to set extensive interactions with the DNA by forming hydrogen, ionic and hydrophobic bonds. Analysis of many plant DNA-binding proteins has led to the identification of a number of highly conserved structural motifs, such as helix turn helix, zinc-finger, helix-loop-helix, leucine and basic-zipper. Homeodomain proteins are a particular class of helix-turn-helix proteins [1].

The interaction between *cis*- and *trans*-acting factors plays a key role in determining the development program in plants as well as in other eukaryotic organisms. This program consists

of a precise spatial and temporal pattern of gene expression, influenced by external agents in the plant kingdom. More recently, silencing led by non-coding micro-RNAs has been described as an additional mechanism of post-transcriptional regulation [2].

Homeobox Genes

Genes containing homeoboxes were initially discovered during the study of homeotic mutants in *Drosophila* and subsequently shown to be present in evolutionary distant organisms, including animals, fungi and plants [3]. The homeobox is a 180 bp consensus DNA sequence present in a number of genes involved in developmental processes. It encodes a 60 amino acid protein motif, the homeodomain (HD), which folds into a characteristic DNA-binding structure composed by three alpha-helices separated by a loop and a turn [4-6]. Compilation of known homeodomain sequences indicates that seven positions are occupied by the same amino acid in more than 95%, ten other positions are conserved in more than 80%, and 12 additional ones present only two amino acids in more than 80%.

These conserved positions define the HD. HD containing proteins act as transcription factors, regulating the expression of target genes by specific interaction with *cis*-elements present in their promoters or other regulatory sequences [7-8].

Plant Homeobox Genes

In plants, the first homeobox identified was Knotted1 (Kn1), a maize gene for which dominant mutations affect leaf development [9]. The knotted leaf phenotype is due to the ectopic expression in leaves of the Kn1 gene, whose activity is normally restricted to meristematic cells [10]. Additional Kn1-like genes (termed knox genes) have been isolated from maize and other monocot and dicot species, indicating that this class of genes constitutes a family present throughout the plant kingdom [11-14].

From kn1 isolation up to now, genes encoding homeodomains were identified in a wide range of plant species including monocots and dicots. They can be divided and subdivided into different families and subfamilies according to sequence conservation in and outside the HD and other conserved domains [9,15-24].

Plate I shows the prototype of each family including protein tail, location of the homeodomain and other conserved domains. Up to this moment, the following eight families have been identified: knotted, glabra/HD-Zip IV, Bell, PHD, Zmhox-PHD, HD-Zip, WUS and FWA. Most of the families have been named after the first identified member.

The Association of a Homeodomain with a Leucine Zipper is Unique to Plants

Homeodomain-leucine zipper (HD-Zip) proteins constitute one of the homeodomain-containing transcription factors families. These proteins are characterized by the presence of a specific DNA-binding domain, the homeodomain, associated to an adjacent dimerization motif, the leucine zipper. Both, HDs and LZs are common motifs present by themselves in other eukaryotic kingdoms, such as animals or fungi. However, association of these two motifs in a single transcription factor is apparently a characteristic unique to plants. This family of proteins can be divided into several subfamilies according to sequence homology, gene structure and function.

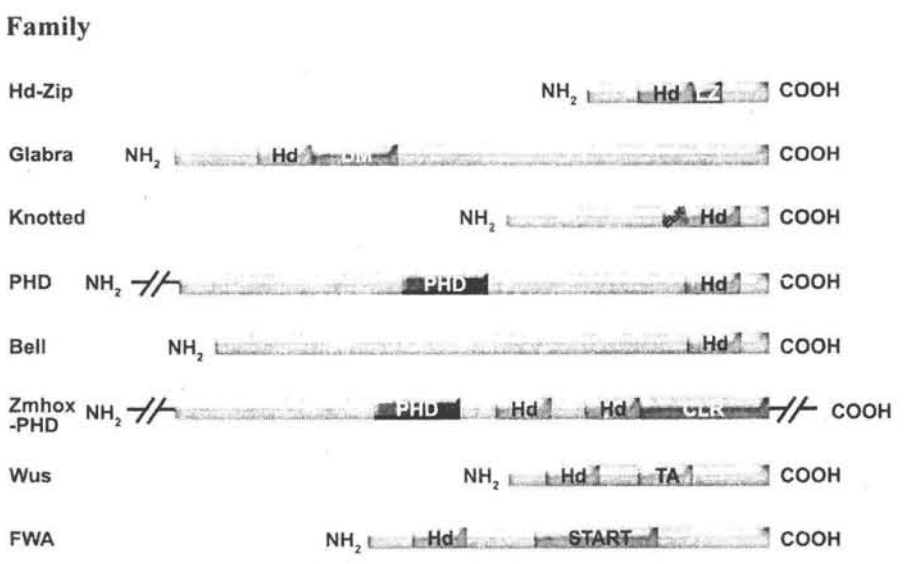

Plate I. Scheme of representative members of each family of plant homeodomain-containing transcription factors. Eight families are represented in the figure: knotted, glabra/HD-Zip IV, Bell, PHD, Zmhox-PHD, HD-Zip, WUS and FWA. Hd: homeodomain; LZ: leucine zipper; DM: dimerization motif; ELK: ELK domain; PHD: PHD finger; CLR: collagen-like repeat; TA: transactivation domain; START: lipid binding start domain.

There are four classes of HD-Zip proteins, each of which is composed of several members from different plant species [16].

It has been suggested and subsequently supported by experimental evidence that HD-Zip proteins are involved in the regulation of developmental processes associated with the response of plants to environmental conditions [16,25-40].

Structure and Function of HD-Zip Proteins

Homeobox genes encode a homeodomain, a sixty amino acid protein motif that interacts specifically with DNA [4-6,41]. The homeodomain folds into a characteristic three-helix structure. Helix I and II are connected by a loop, while helix II and III are separated by a turn which makes this region of the homeodomain bear a resemblance to prokaryotic helix-turn-helix transcription factors. Most homeodomains are able to bind DNA as monomers with high affinity, through interactions established by helix III (the so called recognition helix) and a disordered N-terminal arm located beyond helix I [4,7,8,42,43]. However, HD-Zip proteins are only capable of binding DNA when they are dimerized. This dimerization occurs as the result of the interaction of two leucine-zippers [44,45].

HD-Zip proteins belonging to subfamilies I and II are about 300 amino acids long and possess the conserved motifs located in the centre of the molecule. In general, an acidic domain is present in the N-terminal. Members of subfamily I are not conserved outside the HD-Zip and

conservation inside this region, is lower than in members of subfamily II. On the other hand, class II HD-Zip proteins present two external conserved motifs, the CPSCE adjacent downstream the leucine zipper, and a common C-terminal consensus. The CPSCE is responsible for redox cell state sensing [46].

HD-Zip III members are well functionally characterized as development directors of the apical meristem, the vascular bundles and the adaxial domains of lateral organs [47-50] or directors of vascular development [51,52].

HD-Zip IV/glabra proteins constitute a small group of large proteins involved in epidermal cells fate determination and in the regulation of cell layer-specific gene expression. Other members of this family affect anthocyanin accumulation of the leaf subepidermal layer and root identity [53,54].

In addition to the HD-Zip domains, proteins of subfamilies III and IV present a steroidogenic regulatory protein-related lipid transfer domain, proposed to be a binding steroid-like ligand domain [55]. A phylogenetic tree based on HD-Zip known sequences has been constructed and is shown in Figure 1.

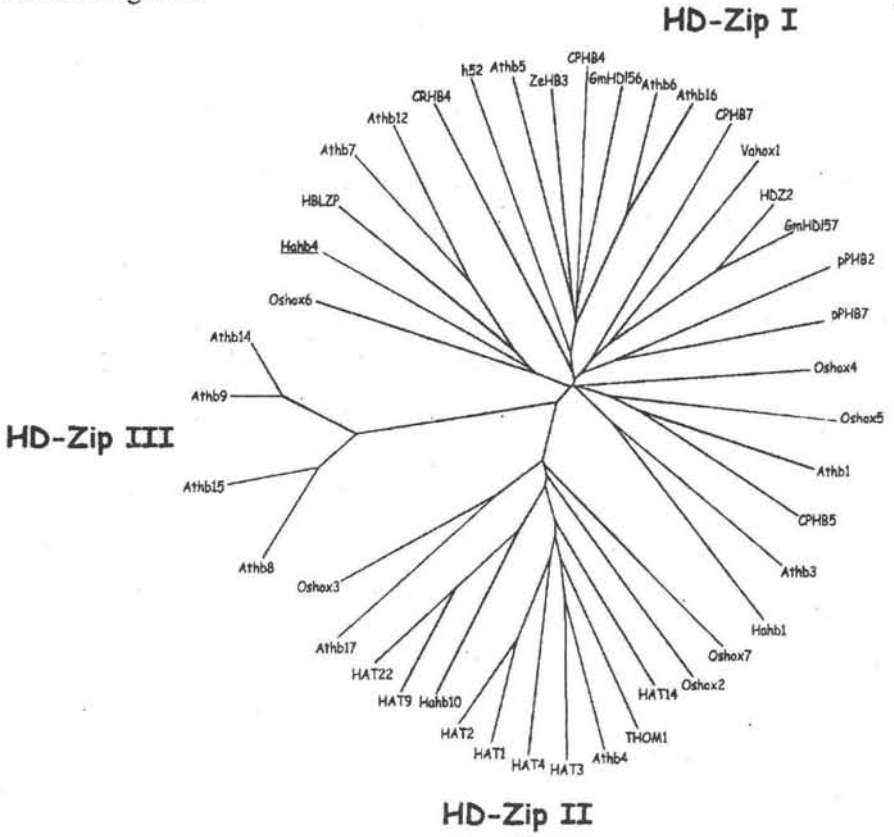

Figure 1. Phylogenetic tree of plant homeodomain-leucine zipper sequences. The analysis was performed using programs from the PHYLIP group [68] on an alignment of plant HD-Zip sequences generated by ClustalW. The tree shown is a consensus one generated after bootstrap analysis of 100 trees performed with Protdist followed by Neighbor.

HD-Zip Proteins Binding Specificity

A large group of homeodomains recognizes the sequence TAATNN as monomers, although other recognition specificities have been observed. Structural studies of homeodomain-DNA complexes have indicated that specific contacts with DNA are established by residues present in the third helix and in the disordered N-terminal arm. Some homeodomain proteins form complexes with each other, which increases the specificity and the affinity of the interaction with DNA.

The removal of the leucine-zipper or the introduction of extra amino acids between the zipper and the homeodomain causes a complete loss of binding, indicating that the relative orientation of the monomers is essential for an efficient recognition of DNA [56,57]. A schematic representation of the interaction between an HD-Zip protein and its DNA target sequence is shown in Plate II.

Proteins that belong to the HD-Zip I and II subfamilies recognize a 9-bp dyad symmetric sequence of the type CAAT(N)ATTG, which can be regarded as composed of two partially overlapping TNATTG sequences [45]. It has been postulated that each monomer interacts with one of these half-sequences in a way that resembles the interaction of monomeric animal homeodomains with DNA. HD-Zip I and II proteins prefer different nucleotides at the central position of the recognition sequence (i.e. A/T and G/C, respectively). The specificity for binding at the central position seems to be conferred in part by amino acids 46 and 56 of helix III (Ala and Trp in HD-Zip I; Glu and Thr in HD-Zip II), together with a different orientation of the conserved Arg55 in both proteins, which would be directly responsible for the interaction [58]. Proteins belonging to subfamily III interact with the target sequence GTAAT(G/C)ATTAC [59] whereas members of subfamily IV/glabra do so with the sequence CATT(A/T)AATG [60].

Hydroxyl radical footprinting protection and interference techniques have been employed to analyze the interaction of the sunflower HD-Zip proteins Hahb-4 and Hahb-10, which belong to classes I and II, respectively, with target sites containing A/T or G/C base pairs at the central position. The results are indicative of a opposite orientation of each homeodomain that form the dimer, respective to the TNATTG half-sequence it binds. The nucleotide present at the central position of each strand in both target sites would be in part responsible for this behavior [61].

Regulation of HD-Zip Encoding Genes

Concerning *Arabidopsis thaliana,* it has already been possible to identify 17 members of the subfamily I and 9 of the subfamily II. These genes are distantly related to the subfamilies III and IV members and share a common origin. They are not as well functionally characterized as other plant homeodomain containing transcription factors but the available information indicates that they would mediate in mediating the effects of environmental conditions to regulate growth and development in plants [62]. Examples of such regulation are given in Table 1.

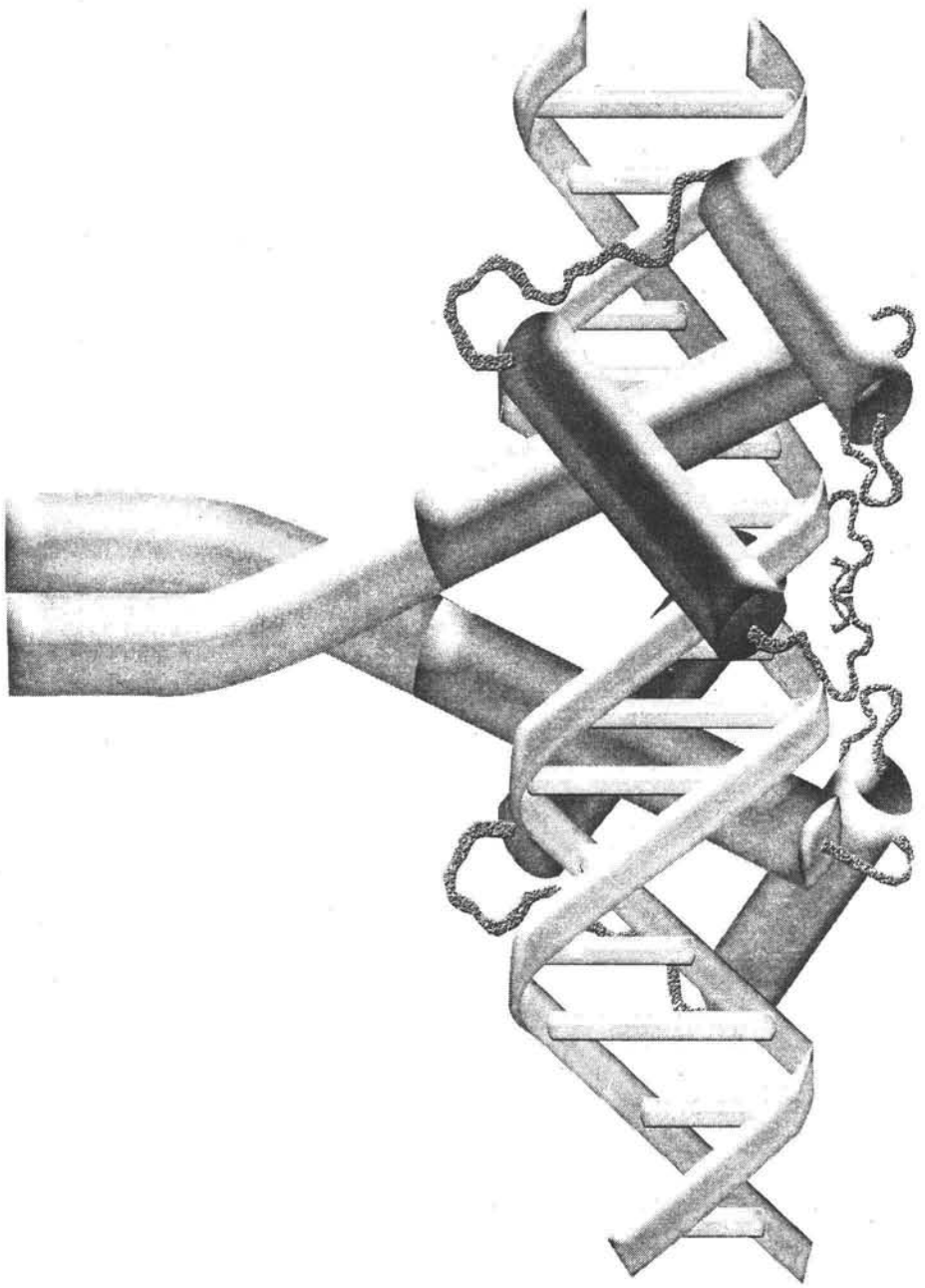

Plate II. Schematic representation of the interaction between an HD-Zip protein and its target DNA sequence. DNA colored in yellow, leucine-zipper in pink, helix III of the HD in green, helix I of the HD in purple, and helix II of the HD in light blue.

Table 1. Examples of HD-Zip encoding genes regulated by abiotic agents

HD-ZIP	*Subfamily*	*Plant*	*Regulating agent*
Athb2/HAT 4	II	*Arabidopsis thaliana*	Light
Hahb-10	II	*Helianthus annuus*	Light
Athb1/Athb16	I	*Arabidopsis thaliana*	Light, salt, cold (repressed)
Athb13	I	*Arabidopsis thaliana*	Sugar signaling
Athb 7/Athb12	I	*Arabidopsis thaliana*	ABA, salt, cold
Hahb-4	I	*Helianthus annuus*	ABA, drought, salt
Athb6/Athb 40/Athb 53	I	*Arabidopsis thaliana*	ABA, salt, cold
Athb5/Athb 21	I	*Arabidopsis thaliana*	ABA, salt
Athb52	I	*Arabidopsis thaliana*	cold (repressed)

Similar but not identical roles in light responses were described for *ATHB2/HAT4*, from *Arabidopsis thaliana* and *Hahb*-10, from sunflower, both members of the subfamily II [32,34]. Moreover, *ATHB1* and *-16* belonging to subfamily I were also proposed to be regulators of different developmental events in response to light quality and intensity. *ATHB13* was proposed to be a potential mediator of sugar signaling, whereas a good number of the same subfamily (I) members have been proposed to be involved in abscisic acid (ABA) related responses (*ATHB7*, *-12*, *-6*, *-5*). All of these genes are either up or down regulated by water deficit conditions [31,36,62].

Treatment of *Arabidopsis* plants with ABA or NaCl resulted in an up-regulation of *ATHB*-7 and -12 transcript levels by a factor of 12-25 times in relation to the untreated control. Expression of *ATHB6*, *-21*, *-40*, and *-53* also increased after these courses of treatment but by a lower factor (approximately 2 fold) in comparison to the levels measured in control grown plants. Repression of *ATHB3*, *-23*, *-5* and *-52* to approximately the half of its normal expression was observed in the same treated plants.

ATHB1 and *-16* had their transcript levels reduced when plants were treated with salt but not in response to ABA. Low temperature exposure produces up regulation of *ATHB6*, *-7*, *-12*, *-40* and *-53* (2-4 fold), while *-1*, *-16* and *-52* expression was reduced under the same conditions to the half of its normal expression. Other HD-Zip I genes were unaffected by these treatments but its expression was altered by different light conditions [62].

The results presented by Henriksson *et al.* [62], in addition to previously reported ones indicate that the majority of the HD-Zip I genes are responsive to one of the external conditions applied including ABA, water deficit stress, and light. In other species like sunflower, rice and barley, several HD-Zip encoding genes were isolated and turned out to be regulated by abiotic stress, mediated or not by ABA [28,33,63].

Expression analysis together with plant transformation with this type of genes offered experimental support to the initial theory introduced by Schena and Davis [22], which suggest that HD-Zip proteins regulate plant development in response to environmental conditions.

Participation of HD-Zip Proteins in Response to Abiotic Stress

Induction or repression of a certain transcription factor expression by external abiotic stress factors, like drought or salinity, indicates that the gene is somehow involved in the response to

the external stimuli but does not indicate how. Transcription factors play different roles, as activators or repressors of a great number of target genes participating in a wide range of metabolic pathways.

Aiming to determine which role plays a particular transcription factor, essentially two strategies have been used: working with mutants and transforming plants, so as to overexpress or suppress the gene activity. The first strategy is only applicable to plants whose genomic information and mutants libraries are available, like the model *Arabidopsis thaliana*. The second strategy is based on the use of a transformable plant, for which heterologous systems are limited to overexpression, since antisense technology needs a rank of nucleotide sequence homology that does not exist among species.

Overexpression experiments have been carried out with a reduced number of HD-Zip genes responding to abiotic stress. In this sense, *Athb*-7 and -12 were overexpressed in *Arabidopsis* resulting in phenotypic altered plants that did not show any enhanced stress tolerance [64]. Hahb-4 is the sunflower HD-Zip protein most related to ATHB7 and -12, being the three of them, together with Oshox6 from rice [63] and HPLZP from *Prunus americana* (Accession Number AF139497) in the same branch of the phylogenetic tree [28]. However, overexpression of sunflower *Hahb*-4 led to obtain drought-tolerant *Arabidopsis* plants which only share with Athb-7 and -12 overexpressing transgenic plants some morphological characteristics, as well as a marked delay in development [65]. In spite of these undesired characteristics observed, once stress tolerance was achieved, improvement of the biotechnological system was possible. In the case of *Hahb*-4, changing the constitutive promoter 35S CaMV used in preliminary essays by a stress-inducible one resulted in transgenic plants with enhanced stress tolerance whose phenotype is indistinguishable from that of the wild type ones [66-68].

All the experimental results obtained up to now indicate that HD-Zip coding genes may be excellent biotechnological tools under the control of inducible promoters instead of constitutive ones.

Future Perspectives

HD-Zip coding genes are involved in plant development in response to environmental factors. This fact lets us to consider them to be used as tools to improve agronomical crops. For instance, *HAT4* and *Hahb*-10 overexpression produce an acceleration of growth rate, while *Hahb*-4 overexpression produces drought-tolerant plants when *Arabidopsis* is taken as an heterologous system. Obviously, further studies are still required to evaluate whether this genes really confer these characteristics to species of agronomic value. Nowadays, the development of improved plant varieties, by transforming plants with HD-Zip encoding genes represents a very promising task. The identification of these genes, followed by structural and functional characterization, conforms the first step of this biotechnological strategy. At the same time it is necessary to isolate and characterize suitable inducible promoters and combine them with the adequate genes to reach the objective.

Concluding Remarks

In conclusion, the knowledge about the processes of gene expression in response to abiotic stress keeps enlarging, and it would be of great consequences if more efforts were invested in

both, breeding and genetic manipulation programs. Identification of transcription factors, able to switch defense responses, will contribute to obtain potential biotechnological tools. In this sense, HD-Zip proteins appear to be good candidates to confer tolerance to different abiotic stresses. Research on this field ought to be taken further, including also proteins from a larger number of plant species apart from the usual models. A complete characterization of promoters and the analysis of their activities in transgenic plants will be vital to achieve success. Physiological studies combined with molecular research will aid to a better comprehension of the system as a whole. Proper research will enable humanity to satisfy the increasing food demand by the achievement of highly productive crops.

Acknowledgements

Work carried out by our research group was supported by grants from CONICET, ANPCyT (Agencia Nacional de Promoción Científica y Tecnológica), Fundación Antorchas and Universidad Nacional del Litoral. RLC is a member of CONICET (Argentina), FDA and PAM are fellows of CONICET and ANPCyT (PAV 137/2/2) respectively.

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2 Prospects for Crop Improvement through Photosynthesis by Flag Leaf Rolling in Spring Wheat: Effect of High Irradiance

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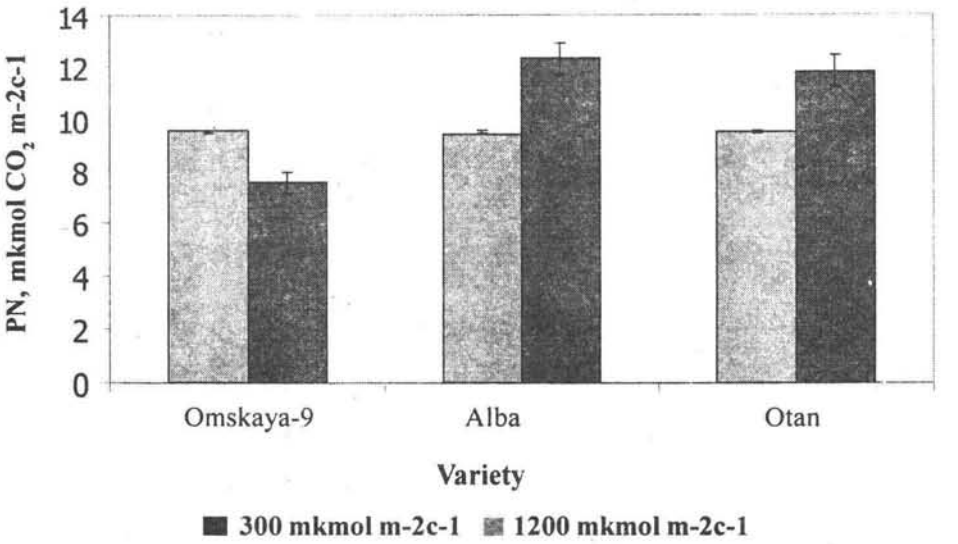
Increasing the maximum yield potential is viewed as an important part of any strategy for achieving crop improvement [1]. An increase in yield potential will have to involve an increase in crop biomass, i.e. there will have to be more net photosynthesis [2]. This may be achieved by an increase in leaf area index or an increase in net photosynthesis per unit leaf area.

Challenge is worthwhile considering the very substantial harmful impact of drought on crop production. The phenomenon of drought is complex in itself [3]. To plant, drought is an interaction between precipitation, irradiance, evapotranspiration, soil physical properties, soil nutrient availability etc.

Genetically complex mechanisms of drought resistance include mechanisms of escape, drought avoidance and drought tolerance. Little is known about the process of acclimation of photosynthesis to irradiance. Therefore our emphasis were placed upon the study of mechanisms of drought avoidance by mean of morphological features particularly flag leaf rolling trait. Several studies have assessed the contribution of leaf rolling to maintenance of turgor and increased water use efficiency [4], but have not considered its potential for protecting the photosynthetic apparatus from action of light. It is shown that for wheat the flag leaf rolling display's as a diurnal response to an increase in solar irradiation [5].

In our investigation, spring wheat near isogenic lines, Otan (BC5) and Alba (BC3) with inserted flag leaf rolling trait (*Rl*1 and *Rl*2) having higher yield in drought conditions compared to non-rolling cultivar, Omskaya 9 were used [6]. Plants were grown in green house for 80 days under conditions of moderate (300 μmol $m^{-2}s^{-1}$) and high (1200 μmol $m^{-2}s^{-1}$) light intensity. We determined the rate of photosynthesis (P_{max}), parameters of gas exchange *in vivo* (stomatal conductance gH_2O, μmol $m^{-2}s^{-1}$), chlorophyll fluorescence (Fv/Fm, Fv/Fo, RFd 690) and an index of stress adaptation of photosynthetic apparatus.

Figure 1 shows the light-saturated rates of photosynthesis (P_{max}) for Otan, Alba and Omskaya 9 measured using an integrated light-emitting diode as light source. P_{max} declined in



Figutr. 1. The light-saturated rates of photosynthesis (P_{max}) expressed per unit leaf area for flag leaf in different genotypes of wheat, Otan, Alba and Omskaya 9 measured using an integrated light-emitting diode as light source. Moderate and high irradiance are 300 μmol $m^{-2}c^{-1}$ and 1200 μmol $m^{-2}c^{-1}$, respectively.

Omskaya 9 under high irradiance compared to moderate irradiance. For flag leaves of Otan and Alba grown at high light intensity P_{max} was 24% and 30% higher then that in plants under moderate irradiance, correspondingly.

Stomatal closure is an important adaptation to drought, but it restricts CO_2 supply to the chloroplasts and by reducing the fraction of incident energy that can be used for carbon reduction predisposes the photosynthetical apparatus to photoinhibitory damage by high light [7]. The analyses of data of stomatal conductance measurements on upper side of flag leaf indicate that there were significant differences between nonrolling and rolling genotypes of wheat grown under high irradiance (Figure 2). The light regime did not affect on the stomatal conductance in cultivar Omskaya 9. For Otan and Alba lines stomatal conductance increased on 45% and 77% compared to that in plants under moderate light intensity, correspondingly.

The correlation between stomatal conductance and the rate of photosynthesis for the Omskaya 9 ($r^2 = 0.59$) was lower then for Otan ($r^2 = 0.99$) and Alba ($r^2 = 0.89$) suggesting involvement of other factors to limitation of photosynthesis at high irradiance. In rolling lines, an increase in the rate of photosynthesis related to enhanced CO_2 supply as indicate our measurements of internal content of CO_2 in flag leaf (data are not shown).

It is important to assess the operational photosynthetic characteristics of the flag leaf of wheat lines differing in rolling. Chlorophyll fluorescence was used to measure the efficiency of Photosystem II electron transport during moderate and high irradiance grown conditions. The measurement of fluorescence characteristics shows that there were no differences in the ratios of Fv/Fm and Fv/Fo at both light intensities between genotypes. However strong difference was seen in another fluorescence parameter: the rate of fluorescence decrease measured at 690

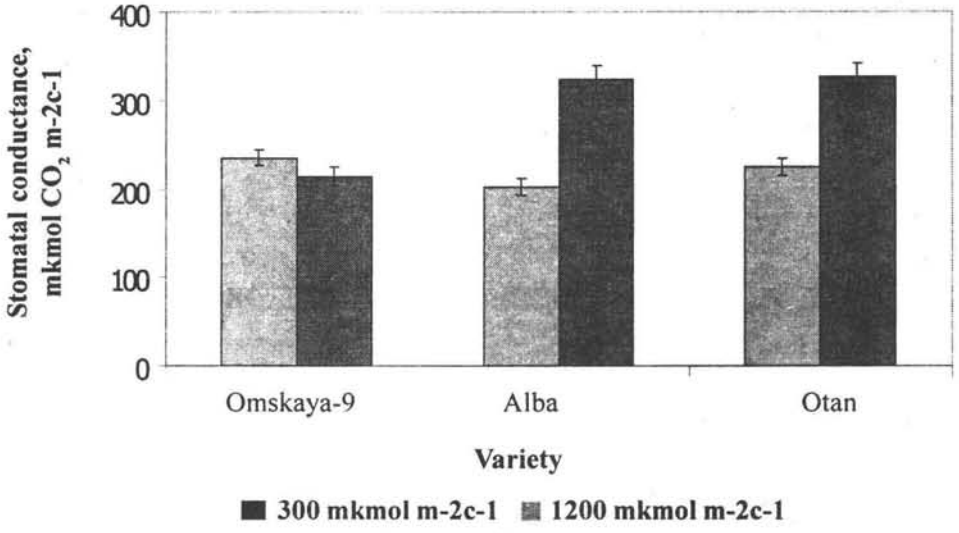


Figure. 2. The stomatal conductance on upper side of flag leaf of nonrolling (Omskaya-9) and rolling genotypes (Otan and Alba) of wheat grown under moderate (300 μmol $m^{-2}c^{-1}$) and high (1200 μmol $m^{-2}c^{-1}$) irradiance.

nm (RFd 690). The change in this parameter is a good indicator of potential photochemical activity of Photosystem II [8]. We found that RFd 690 increased in both rolling genotypes with increasing irradiance (Figure 3). In contrast, the RFd 690 value for nonrolling cultivar, Omskaya 9 decreased in high light grown conditions. Thus, the close relationship between the rate of

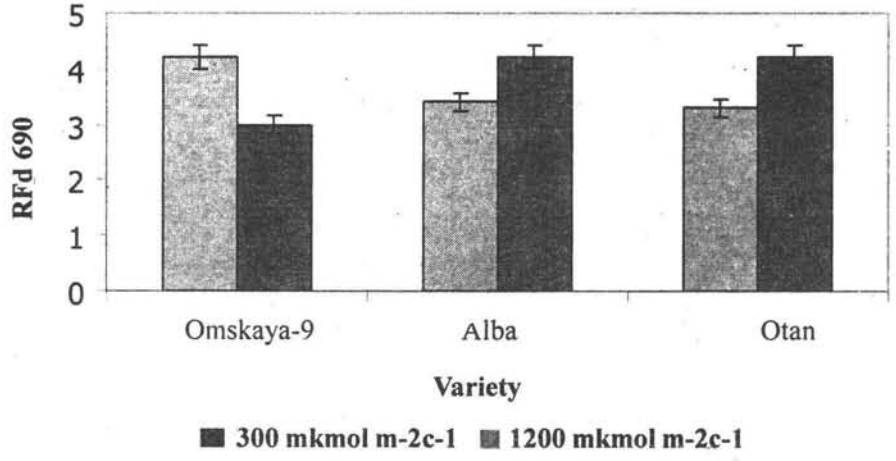


Figure. 3. The chlorophyll fluorescence RFd 690 in flag leaf of nonrolling (Omskaya-9) and rolling genotypes (Otan and Alba) of wheat grown under moderate (300 μmol $m^{-2}c^{-1}$) and high (1200 μmol $m^{-2}c^{-1}$) irradiance.

photosynthesis and intensity of chlorophyll fluorescence RFd 690 indicates that an appearance of flag leaf rolling prevents photosynthetical apparatus from photoinhibition, induced by high irradiance.

The ability of leaf to acclimate to increasing irradiance was also estimated by determination of the stress adaptation index Ap, which is determined from the RFd-values of chlorophyll fluorescence induction kinetics [9]. This allows to estimate the capacity of photosynthetical apparatus to structural and functional changes under unfavorable conditions. The Ap-values of nonrolling Omskaya 9 decreased at high light intensity growth. However in genotypes having rolling trait grown at high irradiance, the Ap-indexes were higher then that in moderate irradiance grown plants. This fact shows a good adaptation capacity of photosynthetical apparatus in leaf rolling genotypes.

In conclusion, the investigation of the mechanisms of drought avoidance by leaf rolling trait in aspect of photosynthetic performance allows us to show an advancement of this morphological trait to high irradiance. We have shown clear differences in photosynthetic performance between nonrolling and rolling wheat genotypes. The flag rolling wheat lines have strong acclimation capacity of photosynthesis to light regime. High irradiance induced the rate of photosynthesis in rolling leaf. Acclimation of photosynthesis to irradiance is conditioned by minimizing photoinhibition. Thus the leaf rolling trait as an effective protective mechanism from the effects of high irradiance results in an optimization of the proportion of absorbed versus utilized photons in Photosystem II. It is also shown the important physiological role of stomatal regulation in photosynthesis of leaf rolling lines.

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3 The Changes in Extreme High-temperature Tolerance and Antioxidant System of *Nelumbo Nucifera* Seeds

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Introduction

Sacred lotus (*Nelumbo nucifera*) has been a prestigious crop in China for nearly 5000 year, their fruits are of extreme longevity [6, 22, 26]. The oldest cultivated fruit germinated and directly radiocarbon dated is that of a sacred lotus >1000 yr old [26], and 200-1300-year-old sacred lotus fruit have an overall germination percentage of 80% [27]. It has been suggested that the extreme longevity of sacred lotus fruit might be related to (1) protection role of pericarp and heat-resistant proteins, (2) repair role of protein-repair enzyme, L-isoaspartyl methyltransferase [25]; and (3) higher polyunsaturated fatty acid [22].

Another outstanding character of sacred lotus fruits is their high-temperature tolerance. Ohga [20], in a 1500-fruit study of *China Antique* from the Xipaozi lakebed, observed 50% germination after incubation of intact fruit in water for 2 h at 90°C, and 60% germination in old Xipaozi fruits subjected to water at 80°C for ~4 d. However, the results by Tang [29] also indicated that the high-temperature tolerance of sacred lotus seeds was higher that of their fruits, and that the pericarp had a negative effect in high-temperature tolerance.

In plants, reactive oxygen species (ROS) are continuously produced predominantly in chloroplasts, mitochondria, and peroxisomes. Production and removal of ROS must be strictly controlled. However, the equilibrium between production and scavenging of ROS may be perturbed by a number of adverse abiotic stress factors such as light stress, water stress, temperature stress, oxidative stress, and so on [2, 9, 10]. Up to now, the most frequently cited cause of seed deterioration is lipid peroxidation. Lipid peroxidation begins with the generation of a free radical either by autoxidation or enzymatically by oxidative enzymes such as lipoxygenase present in many seeds [16]. In the present study, sacred lotus fruits from Hekou of Yunnan, China were used as experimental materials, we have focused on extreme high temperature tolerance of sacred lotus seeds and the changes in antioxidant enzyme (superoxide

dismutase [SOD], ascorbate peroxidase [APX], catalase [CAT], and glutathione reductase [GR]) activities and ROS (H_2O_2 and superoxide radical [$\cdot O_2^-$]) and thiobarbituric acid (TBA)-reacting substance contents during extreme high temperature.

Materials and Methods

Plant Materials

Sacred lotus (*Nelumbo nucifera* Gaertn.) fruits were collected at maturity in Hekou of Yunnan, China in September, 2001. After taking out from seedpot of the lotus, fruit were cleaned in water, and then dehydrated for 30 days at 25±2°C, 70% relative humidity (RH) and to a water content of 0.103±0.003 g H_2O/g DW, and then kept at 4°C until used. Seeds of maize (*Zea mays* L. Yuedan 1) and mung bean [*Vigna radiate* (Linn.) Wilczek Sulv 1] were obtained from South China Agricultural University, Guangzhou of China, and were kept at 4°C until used.

Sacred lotus seeds removed pericarp, maize and mung bean seeds, were treated at 100°C for different time, and then used as assay.

Water Content Determinations

Water content of sacred lotus axes and cotyledons, maize embryos and endosperms, and mung bean seeds was determined gravimetrically (80°C for 48 h). Water contents are expressed on a dry mass basis (g H_2O/g DW, g/g).

Germination Assessment

Batches of 25 seeds for sacred lotus, 50 seeds for maize and mung bean, were germinated on moist filter paper moistened with 10 ml of distilled water in closed Petri dishes for 5 days at the dark and 25±1°C. Seeds showing radicle emergence for 2 mm were scored as germinated.

Conductivity Tests

Relative leakage of 5 axes of sacred lotus seeds was measured using a Model 4310 conductivity meter (Jenway Lit, Essex, England). 5 axes were placed in 4 ml distilled water, conductivity of leakage was measured immediately (A_0), and then kept at 25±1°C and shaken for 5 times, after 2 h, conductivity of leakage was secondly measured (A_1), finally these axes were boiled at 100°C for 30 min and cooled in tap water, conductivity of leakage was again measured (A_2). The relative leakage = $(A_1 - A_0) / (A_2 - A_0) \times 100\%$.

Chlorophyll Content Determinations

After sacred lotus seeds removed pericarp were treated at 100°C for different time, the chlorophyll content of 20 axes was immediately measured according to method of Arnon [3].

Ultrastructural Studies

Hypocotyls of sacred lotus seeds treated at 100°C for different time were fixed for 24 h in 3% glutaraldehyde in 50 mM sodium cacodylate buffer. After rinsing, the material was fixed for

24 h in 0.5% aqueous osmium tetroxide followed by routine dehydration and embedding. Sections were stained with lead citrate, then viewed and photographed with a JEM-1230 (JEOL LTD) transmission electron microscope.

Assay of SOD

Forty axes and cotyledons of sacred lotus seeds were homogenized to a fine powder with a mortar and pestle under liquid nitrogen, respectively. Subsequently soluble proteins were extracted by grinding the powder in an extraction mixture composed of 50 mM phosphate buffer (pH 7.0), 1.0 mM ethylenediaminetetraacetic acid (EDTA), 0.05% (v/v) Triton X-100, 2% (w/v) polyvinylpolypyrro-lidone (PVPP) and 1 mM ascorbic acid (AsA). The homogenate was centrifuged at 16000 *g* for 15 min, after which the supernatant was transferred to a new tube and kept at -60°C.

SOD (EC 1.15.1.1) activity assay was based on the method of Beauchamp and Fridovich [4], who measure inhibition of the photochemical reduction of nitroblue tetrozulium (NBT) at 560 nm, modified as follows. The 1 ml reaction mixture contained 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 75 μM NBT, 16.7 μM riboflavin and enzyme extract (ca 25 μg protein). Riboflavin was added last and the reaction was initiated by placing the tubes under 15-W fluorescent lamps. The reaction was terminated after 5 min by removal from the light source. An illuminated blank without protein gave the maximum reduction of NBT, and therefore, the maximum absorbance at 560 nm. SOD activity (mean of five replicates) is presented as absorbance of sample divided by absorbance of blank, giving the percentage of inhibition. In this assay, 1 unit of SOD is defined as the amount required to inhibit the photoreduction of NBT by 50%. The specific activity of SOD was expressed as unit mg^{-1} protein.

Assays of APX, CAT and GR

A fine powder of 60 axes and cotyledons of sacred lotus seeds homogenized in a mortar under liquid nitrogen was extracted by grinding in 5 ml of 50 mM Tris-HCl (pH 7.0), containing 20% (v/v) glycerol, 1 mM AsA, 1 mM dithiothreitol, 1 mM EDTA, 1 mM reduced glutathione (GSH), 5 mM $MgCl_2$ and 1% (w/v) PVPP. After two centrifugation steps (12,000 *g* for 6 min and at 26,900 *g* for 16 min respectively), the supernatant was stored at -60°C for later determinations of enzyme activities of APX, CAT and GR.

APX (EC 1.11.1.7) was assayed as the decrease in absorbance at 290 nm (2.8 mM^{-1} cm^{-1}) due to AsA oxidation, by the method of Nakano and Asada [18]. The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 1 μM AsA, 2.5 mM H_2O_2 and enzyme source (ca 25 μg protein) in a final volume of 1 ml at 25°C.

CAT (EC 1.11.1.6) activity was determined by directly measuring the decomposition of H_2O_2 at 240 nm (0.04 mM^{-1} cm^{-1}) as described by Aebi [1], in 50 mM potassium phosphate (pH 7.0), containing 10 mM H_2O_2 and enzyme source (ca 25 μg protein) in a final volume of 1 ml at 25°C.

GR (EC 1.6.4.2) was determined as the decrease in absorbance at 340 nm (6.2 mM^{-1} cm^{-1}) due to the oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH), according to Halliwell and Foyer [8] in 50 mM Tris-HCl buffer (pH 7.5), containing 5 mM

$MgCl_2$, 0.5 mM oxidized glutathione (GSSG), 0.2 mM NADPH and enzyme extract (ca 25 μg protein) in a final volume of 1 ml at 25°C.

Determination of Superoxide Radical and Hydrogen Peroxide

$\cdot O_2^-$ was measured as described by Elstner and Heupel [7] by monitoring the nitrite formation from hydroxylamine in the presence of $\cdot O_2^-$, modified as follows. Axes and cotyledons of sacred lotus seeds treated for different time at 100°C were homogenized in 6 ml of ice-cold 50 mM sodium phosphate buffer (pH 7.8) at 4°C, and the brei was centrifuged at 12,000 *g* for 10 min. The supernatant was used for determination of $\cdot O_2^-$. The reaction mixture contained 0.9 ml of 50 mM phosphate buffer (pH 7.8), 0.1 ml of 10 mM hydroxylamine hydrochloride, and 1 ml of the supernatant was incubated at 25°C for 20 min. And then 0.5 ml of 17 mM sulfanilamide and 0.5 ml of 7 mM napathylamine were added to 0.5 ml of reaction mixture. After incubation at 25°C for 20 min, the absorption in the aqueous solution was read at 530 nm. A standard curve with nitrite was used to calculate the production rate of $\cdot O_2^-$ from the chemical reaction of $\cdot O_2^-$ and hydroxylamine.

The content of H_2O_2 was measured by monitoring the absorption of titanium-peroxide complex at 410 nm according to the method of MacNevin and Uron [14] and Partterson *et al.* [21], modified as follows. Axes and cotyledons from sacred lotus seeds treated for different time at 100°C were homogenized in 6 ml of 5% (w/v) trichloroacetic acid, and then centrifuged at 12,000 *g* for 10 min. After 1 ml supernatant and 9 ml of 0.2% (w/v) titanium tetrachloride hydrochloride solution were mixed, and the absorbance of reaction solution was measured at 410 nm, and using H_2O_2 as a standard.

Lipid Peroxidation Products

Lipid peroxidation products were determined as the concentration of TBA-reacting substances, as described by Kumar and Knowles [12]. All determinations are means of 5 replications, and content of TBA-reacting substances was expressed as nmol mg^{-1} protein.

Protein Assay

Protein was measured following the procedure of Bradford [5], using bovine serum albumin (BSA) as a standard.

Statistical Analysis

All data were analyzed using a one-way ANOVA model from the SPSS 12.0 package for Windows (SPSS Inc.).

Results

The changes in water content, and germination percentage of sacred lotus, maize and mung bean seeds during high-temperature treatment

Water contents of sacred lotus axes and cotyledons, maize embryos and endosperm, and mung bean seeds were approximately 0.09, 0.104, 0.106, 0.124, and 0.108 g/g, respectively; their water content and seed germination percentage gradually decreased with increasing treatment time at 100°C (Figure 1a, 1b and 2). It was very interesting that germination percentage

of maize and mung bean seeds was zero at 2 h of treatment at 100°C, and of sacred lotus seeds was still 13.5%, by 24 h (Figure 1b). The time in which 50% of sacred lotus seeds have been killed by 100°C treatment (T_{50}) was about 14 h.

Fresh weight of seedling produced by surviving sacred lotus seeds rapidly decreased with increasing high-temperature treatment, for example, fresh weight of seedling treated for 2 h at 100°C decreased by 48% compared with control (Figure 1b).

Changes in Relative Leakage and Total Chlorophyll Content of Sacred Lotus Axes

Relative electrolyte leakage of sacred lotus axes rapidly increased ($P \leq 0.001$) and total chlorophyll content of sacred lotus axes markedly decreased ($P \leq 0.001$) with increasing treatment time at 100°C, for example, relative leakage increased by 184.9%, total chlorophyll content decreased by 37.2% as seeds were treated for 24 h at 100°C (Figure 1c).

Effect of High-temperature Treatment on Subcellular Structure of Sacred Lotus Hypocotyls

After seeds were treated for 0, 8, 12, 16 and 24 h at 100°C, the ultrastructure of hypocotyl cells exhibited marked difference (Figure 3). When seeds were treated at 100°C for less than 12 h, the ultrastructural details of hypocotyls cell were largely maintained with integrated organelles such as numerous mitochondria, normal nuclei and nucleoli (Figure 3C-E). The plasma membrane adpressed against the cell wall except partly separating in the hypocotyls cells treated for 12 h (Figure 3E), and the frequently observed plasmodesmi also shown the metabolic and structural integrity of membrane system. These results implied an apparently normal metabolic state and high-temperature tolerance of seeds treated for 12 h at high-temperature treatment. However, organelles were damaged by high-temperature treatment as seeds were treated for more than 12 h (Figure 3F-H), such as plasma membrane separated from cell wall, endoplasmic reticulum become unclear, nuclei and nucleoli degraded, most of mitochondria swelled, and lipid granules accumulated at cellular periphery. When seeds were treated for 24 h, organelle organization apparently broke-down and deterioration of membranes occurred (Figure 3H).

Changes in Hydrogen Peroxide Content and Production Rate of Superoxide Radical

As shown in Figure 4a, H_2O_2 content in axes ($P \leq 0.100$) and cotyledons ($P \leq 0.001$) decreased during early phase of high-temperature treatment and then increased, and H_2O_2 content was higher in axes than in cotyledons.

Production rate of $\cdot O_2^-$ in axes steeply increased ($P \leq 0.001$), for example, production rate of $\cdot O_2^-$ increased by 455 and 490% in the axes treated for 16 and 24 h than in control axes, respectively; but $\cdot O_2^-$ has not been observed in cotyledons (Figure 4a).

The Changes in Activities of Antioxidant Enzymes

The changes in activities of SOD, APX, CAT, and GR were monitored during high-temperature treatment of sacred lotus seeds (Figure 5). Activities of SOD in axes ($P \leq 0.001$) and cotyledon ($P \leq 0.001$) gradually increased until 16 h of treatment during treatment, and then decreased and SOD activity in axes was higher than in cotyledons (Figure 5a).

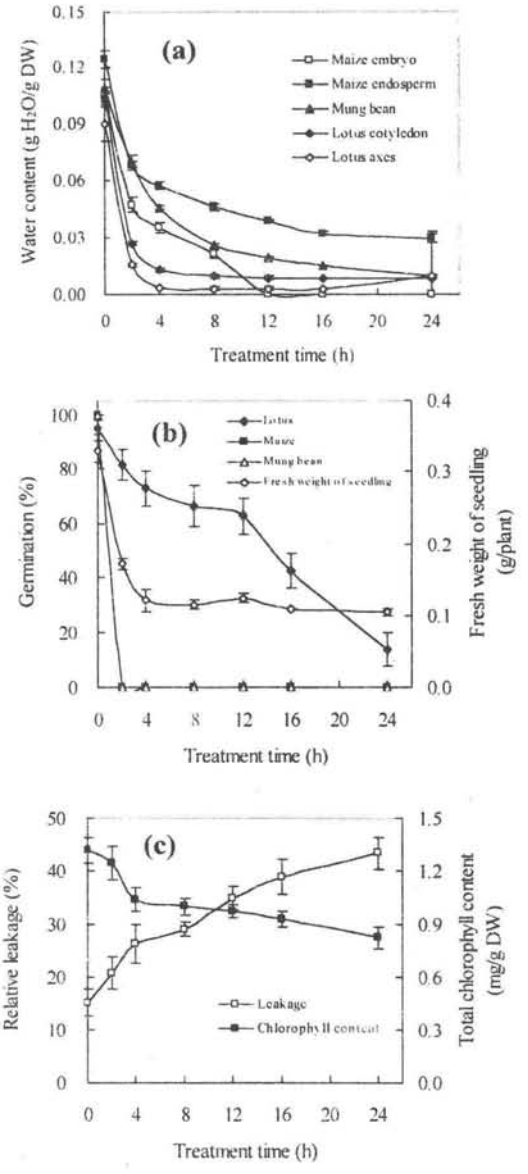

Figure 1. The changes in water content (a), germination percentage, fresh weight of seedling (b), relative leakage and total chlorophyll content (c) during high-temperature treatment of seeds. Seeds were treated for indicated time at 100°C. Water content and germination percentage of seeds, fresh weight of seedling produced by surviving sacred lotus seeds, relative leakage and total chlorophyll content of sacred lotus axes were assayed as described in Materials and Methods. Seeds showing radicle emergence for 2 mm were scored as germinated. Fresh weight of seedling did not include cotyledons. All values are means ± SD of four replicates.

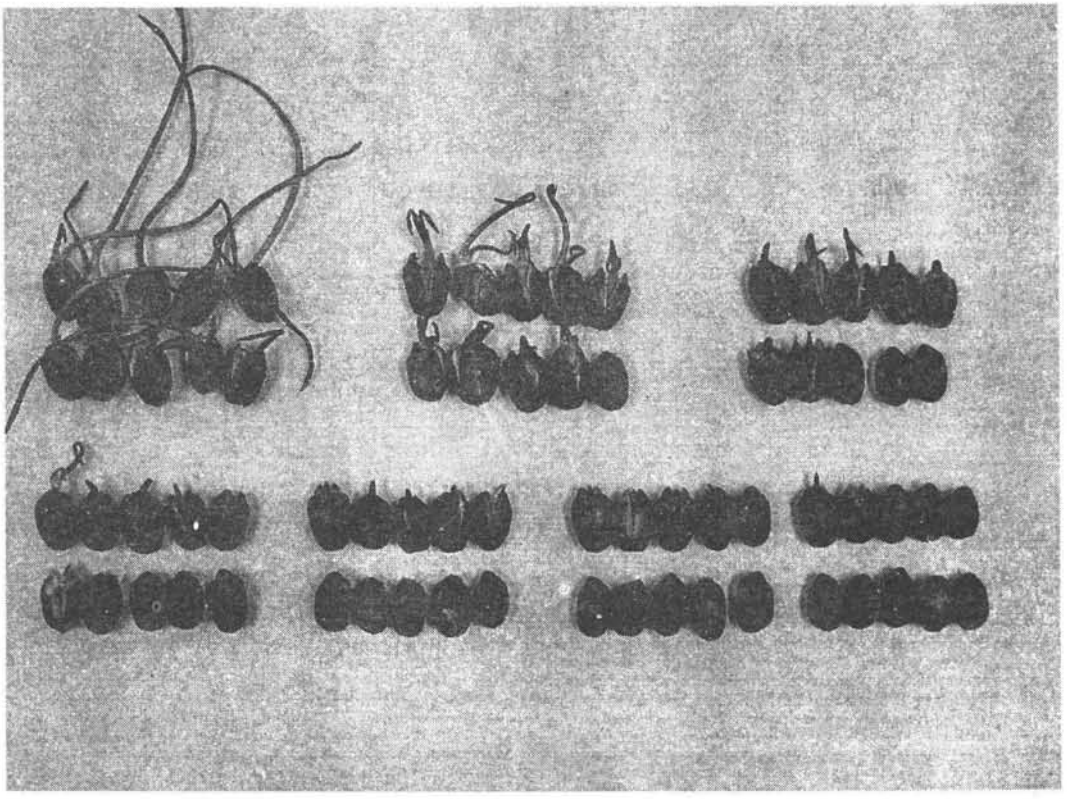

Figure 2. Effects of high temperature on germination of sacred lotus seeds. Seeds were treated for indicated time at 100°C. and were then germinated for 5 days at 25°C.

APX activity of axes progressively decreased with increasing high-temperature treatment time ($P = 0.002$), but one of cotyledons could not be observed (Figure 5a).

During high-temperature treatment, CAT activity in axes gradually increased until 16 h of treatment, and then decreased ($P \leq 0.001$) to approach control level (Figure 5b). However, CAT activity in cotyledons dramatically decreased at the initial 8 h of treatment, for example, CAT activities in cotyledons treated for 4 and 8 h decreased by 71.03 and 85.64%, respectively, compared with control, and then slowly decreased ($P \leq 0.001$). CAT activity was much higher in cotyledons than in axes (Figure 5b).

GR activity in axes firstly increased, and then decreased with increasing treatment time ($P = 0.003$), activity peak of GR was about at 8 h of high-temperature treatment (Figure 4c); but GR activity in cotyledons progressively decreased by the 8 h of treatment, and then hardly any change ($P \leq 0.001$; Figure 5c). GR activity was also much higher in axes than in cotyledons (Figure 5c).

The Accumulation of Lipid Peroxidation Products

TBA-reacting substances are the final products of lipid peroxidation. The content of TBA-

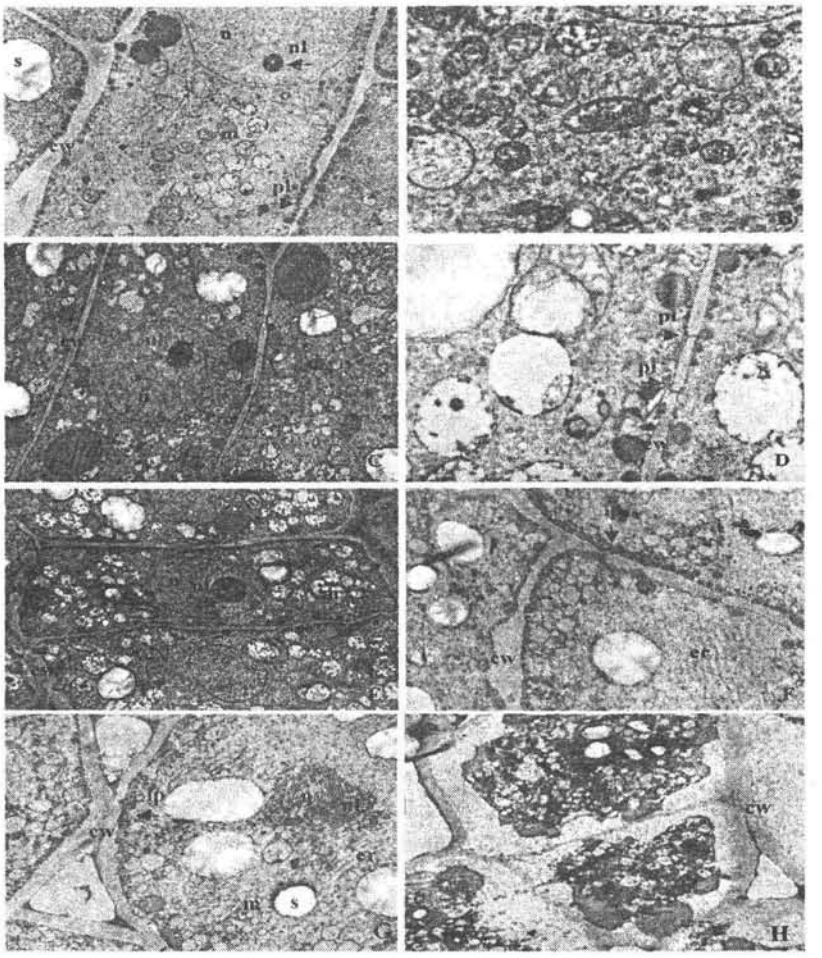

Figure 3. Changes in subcellular structure of sacred lotus hypocotyls. Seed were treated for indicated time at 100°C, and then placed in 100% relative humidity for 48 h at 25°C, and assayed in electron microscopy. A and B, 0 h; show relatively few and small vacuoles, abundant undifferentiated mitochondria (m), integrate nuclei (n) and nucleoli (arrow, nl), starch (s), plasmodesmi (arrow, pl) were also observed (A, 5,000×; B, 20,000×); C, D and E, 8, 8 and 12 h, respectively; show the ultrastructual details were largely maintained, with organelles such as mitochondria (m), nuclei (n) and nucleoli (arrow, nl) and plasmodesmi (pl), and plasma membrane adpressed against the cell wall (cw) except partly seperated in figure E (C, 5,000×; D, 15,000×; E, 5,000×); F, G and H, 16, 16, and 24 h, respectively; in contrast to above cell from seeds treated for less than 12 h, show the damaged organelles, such as plasma membrane separated from cell wall (cw), endoplasmic reticulum (er) with unclear membrane structure, degraded nuclei (n) and nucleoli (arrow, nl), largely dilated mitochondria (m), and peripheral accumulation of lipid (arrow, lp); and apparent break-down of organellar organisation and deterioration of membranes, especially at 24 h treatment (H) (F, 5000×; G, 5000×; H, 5000×).

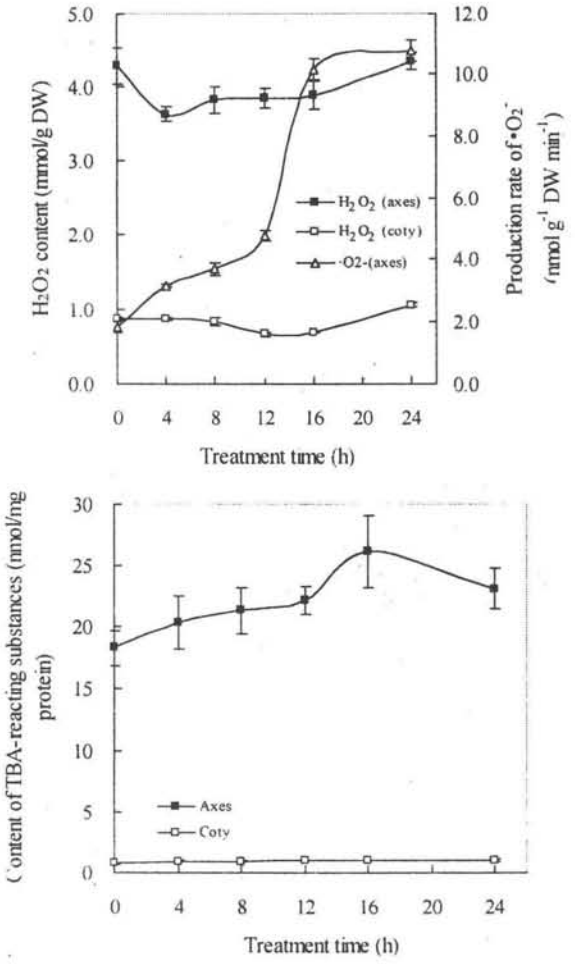

Figure 4. Changes in H_2O_2 content, production rate of $\cdot O_2^-$, and TBA-reacting substances content during high-temperature treatment of sacred lotus seeds. Seed were treated for indicated time at 100°C, and H_2O_2 content, production rate of $\cdot O_2^-$, and TBA-reacting substances content were assayed as described in Material and Methods. All values are means ± SD of five replicates. coty, cotyledon.

reacting substances in axes (P = 0.106) and cotyledon (P = 0.091) gradually increased with increasing high-temperature treatment, and was much higher in axes than in cotyledons (Figure 4b). Although the content of TBA-reacting substances in axes treated for 24 h decreased slightly compared with ones treated for 16 h, they were still higher than that of control axes (Figure 4b).

Discussion

100°C is an extreme high temperature for all life in the world. Germination percentage of maize and mung bean seeds became zero as they were treated for 2 h at 100°C, and of sacred

lotus seeds was still 13.5%, for 24 h (Figure 1b), and T_{50} was about 14 h, showed that maize and mung bean seeds were sensitive, and sacred lotus seeds were much more tolerant to extreme high temperature. We have also confirmed that high-temperature tolerance of sacred lotus seeds was higher than that of their fruits at 100°C, and pericarp had a negative role in high temperature tolerance (data not shown), but its reason is unknown. Pericarp, the hard outer fruit tissue, is not penetrable to water and air [25]. It has been found that water content of sacred lotus fruits was higher than that of seeds within 12 h of high-temperature treatment at 100°C (data not shown), fruits with higher water content could be easily suffered damage under the same high temperature stress than seeds with lower water content. In addition, sacred seeds were highly desiccation tolerance, for example, germination percentage of seeds was still 72.5% as water content of axes treated for 4 h at 100°C was 0.003 ±0.000 g/g.

With increasing treatment time at 100°C, germination percentage of sacred seeds, fresh weight of seedling produced by surviving seeds (Figure 1b), and total chlorophyll content of axes (Figure 1b) gradually decreased, and relative electrolyte leakage of axes (Figure 1c) rapidly increased. These results also showed that sacred seeds were progressively damaged during high-temperature treatment. Ultrastructural observations had given some more direct evidences. When seeds were treated for more than 12 h at 100°C (Figure 3F-H), plasma membrane separated from cell wall, endoplasmic reticulum became unclear, nuclei and nucleoli degraded, most of mitochondria swelled, and lipid granules accumulated at cellular periphery; and organelle organization apparently broke-down and deterioration of membranes occurred as seeds were treated for 24 h (Figure 3H).

H_2O_2 and $.O_2^-$ are synthesized at very high rates in the cells even under optimal conditions. They are produced in copious quantities by several enzymes systems and are involved in virtually all major areas of aerobic biochemistry [2. 17]. H_2O_2 content in axes and cotyledons decreased at early phase of treatment and then increased, and production rate of $\cdot O_2^-$ in axes steeply increased with increasing high temperature treatment (Figure 4a). The chief toxicity of $.O_2^-$ and H_2O_2 is thought to reside in their ability to initiate cascade reactions that result in the production of the hydroxyl radical and other destructive species such as lipid peroxides [19]. A common feature among the different ROS types is their capacity to cause oxidative damage to protein, DNA, and lipids [2].

Enzymatic ROS scavenging mechanisms in plants include SOD, APX and CAT. SOD acts as the first line of defense against ROS, dismutating superoxide to H_2O_2. APX and CAT subsequently detoxify H_2O_2. APX requires an ascorbate and glutathione regeneration system. GR can regenerate GSH from GSSG using NAD (P) H as a reducing agent [2, 16, 19]. Activities of SOD in axes and cotyledon during high-temperature treatment gradually increased until 16 h of treatment, and then decreased (Figure 5a), showing that SOD activity could be induced by high temperature, but was decreased by increasing high-temperature stress dose. These results were similar to findings by Tsang et al. [30], who showed that conspicuous induction of cytosolic CuZn-SOD also takes place under high-temperature treatment in both dark and light conditions. Transgenic tobacco, into which chloroplastic CuZn-SOD has been introduced, shows improved resistance to intense light and low temperature [24]. APX activity of axes progressively decreased with increasing high-temperature treatment time (Figure 5a), indicated that loss of APX activity closely related to loss of germination capacity of seeds. During high-temperature treatment, CAT activity in axes gradually increased until 16 h of treatment, and then decreased

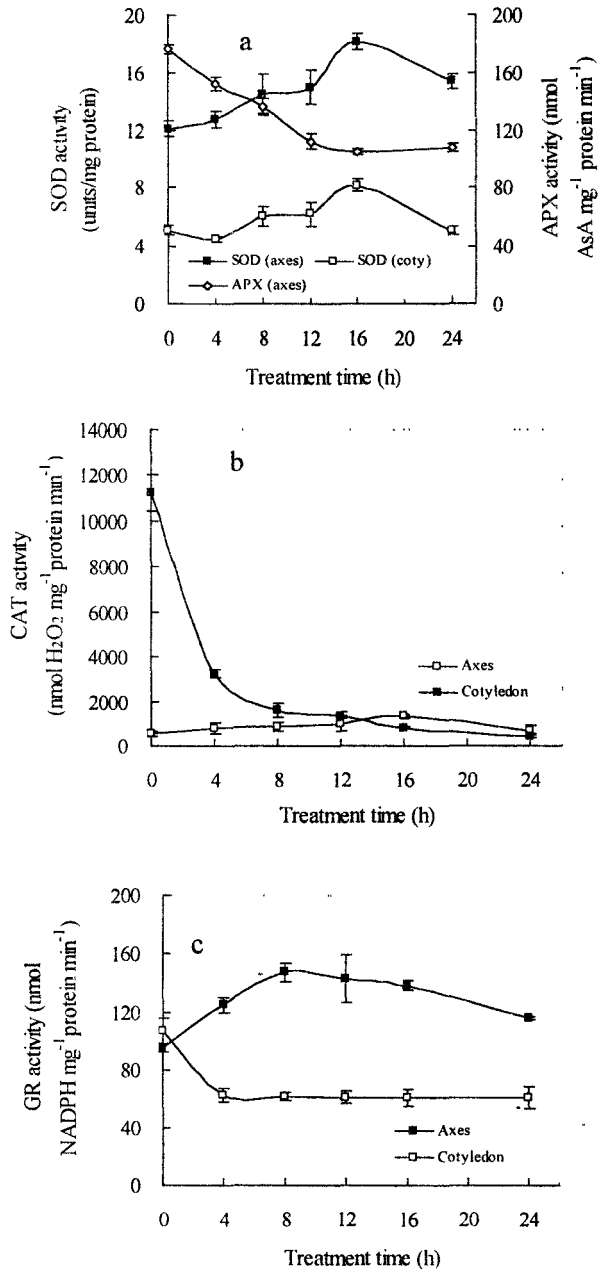

Figure 5. The changes in activities of SOD, APX (a), CAT (b), and GR (c) during high-temperature treatment of sacred lotus seeds. Seed were treated for indicated time at 100°C, and then enzyme activities were assayed as described in Material and Methods. All values are means ± SD of five replicates. coty, cotyledon.

to approach control level (Figure 5b), and CAT activity in cotyledons dramatically decreased at the initial 8 h of treatment and then slowly decreased. CAT is indispensable for oxidative stress tolerance because transgenic tobacco plants with suppressed CAT have enhanced ROS levels in response to both abiotic and biotic stresses [31]. GR activity in axes firstly increased, and then decreased, activity peak was about at 8 h of high temperature treatment, and GR activity in cotyledons progressively decreased (Figure 5c).

TBA-reacting substances are the final products of lipid peroxidation. The content of TBA-reacting substances in axes and cotyledon gradually increased with increasing high-temperature treatment (Figure 4b). Lipid peroxidation has considerable potential to damage membranes and may be a principal cause of seed deterioration [16, 28]. Loss of viability and declining vigour were associated with increase in lipid peroxidation in rapidly aged soybean seeds [11].

SOD (Figure 5a) and GR (Figure 5c) activities, and TBA-reacting substances content were much higher in axes than in cotyledons, and CAT activity was much higher in cotyledons than in axes (Figure 5b), showing that they are of organ specificity.

Sacred lotus fruits (of seeds), which are of extreme longevity and high-temperature tolerance, are excellent materials for studying seed longevity and plant high-temperature stress. Increasing evidence shows that high-temperature tolerance of plant implicates not only in antioxidant systems [9, 10], but also in heat-shock protein [9, 23, 25] especially in small HSP [13, 15]. Studies on proteomics of sacred lotus seeds that are carrying on in our laboratory will further explain their response mechanism to extreme high-temperature.

Acknowledgements

The authors thank the Knowledge Innovation Project of the Chinese Academy of Sciences (KSCX2-SW-117), Hundreds Talent Program of The Chinese Academy of Sciences and Natural Science Foundation of Yunnan (2003C0068M) of China for support of this research. Thanks also to Mr. Qian-fei Xiang and Mrs. Fang Huang (The Section of Seed Biology, Xishuangbanna Tropical Botanical Garden, the Chinese Academy of Sciences) for providing some experimental help.

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4 Myb Transcription Factor Gene Expression in Plant Defense and Stress Responses

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Introduction

Biotic and abiotic stresses are environmental factors that limit plant productivity worldwide [1]. Plants have evolved a range of strategies to increase stress tolerance [2,3]. Different stress responsive genes and different types of transcriptional factors can be used to engineer stress tolerance. Transgenic plants expressing genes such as, betaine aldehyde dehydrogenase (BADH) [4], which catalyses the last step of glycine-betaine synthesis located in peroxisomes in rice and barley [5], improved salt tolerance. Transgenic plants expressing a choline oxidase gene enhanced tolerance to salt stress [6,7]. Transgenic plants expressing a *Capsicum annuum* pathogen and freezing tolerance-related protein 1 (*CaPF1*), which is an ERF/AP2-type transcription factor, increased pathogen and freezing tolerance [8]. Overexpression of two Arabidopsis ERF/AP2 genes, *CBF1/DREBP1B* and *DREBP1A*, resulted in enhanced tolerance to drought, salt, and freezing [9]. Overexpression of a putative R2R3-type MYB transcription factor in Arabidopsis increased low temperature and salt stress, and increased expression of ABA biosynthesis genes during stress [10]. Transcription factors such as ERF/AP2, bZIP/HD-ZIP, Myb, WRKY, each containing a distinct type of DNA-binding domain, have been implicated in plant stress responses in view of the finding that their expression is induced or repressed under different stress conditions [10-12].

Arabidopsis is an important model plant species for plant stress biology [10-12]. Data from the *Arabidopsis* genome project suggest that more than 5% of the genes of this plant encode transcription factors [13]. Genomic analytical approaches have become valuable because it is considered that less than 10% of these factors have been genetically characterized [13,14]. Results of recent studies illustrate some of the challenges to their functional characterization [15]. The availability of the full complement of *Arabidopsis* transcription factors provides the basic framework for future analyses of transcriptional regulation in plants [16-18]. It has been

reported that many transcription-factor gene families exhibit great disparities in abundance among different eukaryotic organisms and that some families are lineage specific [19-21]. The major transcription-factor families of *Arabidopsis* are listed in Table 1. The *Arabidopsis* complement of transcription factors represents a valuable source for other plants. The largest transcription-factor family in *Arabidopsis* is the MYB group, with approximately 180 members

Table 1. Genetically characterized transcription factors in plants

Gene	*Family*	*Species*	*Function*
ABI5	bZIP	*Arabidopsis thaliana*	Abscisic acid response
ANL2	HB	*Arabidopsis thaliana*	Root development, anthocyanin synthesis
ATHB-2	HB	*Arabidopsis thaliana*	Shade-induced growth response
CRC	YABBY	*Arabidopsis thaliana*	Carpel and nectary development
DIC	TCP	*Antirrhinum majus*	Flower development
FAR	MADS	*Antirrhinum majus*	Flower development, C-function gene
FIL	YABBY	*Arabidopsis thaliana*	Development, abaxial-adaxial patterning
Mszpt2-1	Z-C2H2	*Medicago sativa*	Root nodule organogenesis
PIF3	bHLH	*Arabidopsis thaliana*	Light response
REV/IFL1	HB	*Arabidopsis thaliana*	Apical meristem development
SEP1, SEP2, SEP3	MADS	*Arabidopsis thaliana*	Flower development, organ identity
SHP1, SHP2	MADS	*Arabidopsis thaliana*	Fruit dehiscence
SI1	MADS	*Zea mays*	Flower development, B-function gene
WER	MYB	*Arabidopsis thaliana*	Epidermal cell patterning

(Table 1 and 2). Myb regulates phenylpropanoid and lignin biosynthesis (Figure 1; Table 3). A phylogenetic comparison (Figure 2) of a subset of maize and *Arabidopsis* MYB sequences shows that the amplification of this group occurred prior to the separation of monocots and dicots [14,16,22]. However, it has been predicted that maize contains more than 200 MYB genes, and several subgroups appear to have originated recently or undergone duplication [14,16]. At least two clades of MADS-box genes also appear to have been amplified in the phylogenetic lineage that led to grasses [16,23-25]. These informative expansions have provided a functional diversification that might not be present in *Arabidopsis*.

Myb transcription factors play an important role in transcriptional control of plant development and stress tolerance [26-28]. Two flower-specific MYB proteins, AmMYE 305 and -340 from *Antirrhinum* regulate flower development [13,14]. The net activation of transcription of their target genes in any particular cell will be dependent on the relative amounts of the two proteins, their relative abilities to bind DNA and their differing abilities to activate

Table 2. Sequences producing significant alignments after blasting of tomato (*Lycopersicon esculentum*) Myb transcription factor (Myb) protein gene cDNA (AF426174) through NCBI Blast

Species and genotypes	*Locus of gene*	*Score (Bits)*	*E value*
Lycopersicon esculentum blind mRNA	AF426174.1	1243	0.0
Arabidopsis thaliana DNA binding mRNA	NM129245.2	121	5e-24
Arabidopsis thaliana MYB transcription factor	AY519577.1	121	5e-24
Malus x domestica MYB24 mRNA	DQ074472.1	103	1e-18
Arabidopsis thaliana chromosome gene	AC006922.7	91.7	5e-15
Arabidopsis thaliana DNA binding protein	NM119940.2	87.7	8e-14
Arabidopsis thaliana MYB transcription factor	AY519614.1	87.7	8e-14
Arabidopsis thaliana putative protein	AF062914.1	87.7	8e-14
Lotus corniculatus var. *japonicus*	AP004912.1	85.7	3e-13
Arabidopsis thaliana MYB68	NM125976.2	79.8	2e-11
Arabidopsis thaliana MYB transcription factor	AY519647.1	79.8	2e-11
Arabidopsis thaliana At5g65790 gene	BT005994.1	79.8	2e-11
Arabidopsis thaliana putative transcription factor	AF062901.1	79.8	2e-11
Arabidopsis thaliana MYB36	NM125143.2	75.8	3e-10
Arabidopsis thaliana DNA binding protein	NM114829.2	75.8	3e-10
Arabidopsis thaliana mRNA for AtMYB84 R2R	Y14209.1	75.8	3e-10
Arabidopsis thaliana clone 38611 mRNA	AY089148.1	75.8	3e-10
Arabidopsis thaliana MYB55	NM001036494.1	73.8	1e-09
Arabidopsis thaliana BAC T15B16	AF104919.1	73.8	1e-09
Arabidopsis thaliana MYB99	NM_125626.1	71.9	4e-09
Arabidopsis thaliana mRNA for MYB	AK175687.1	71.9	4e-09
Arabidopsis thaliana genomic DNA	AB019235.1	71.9	4e-09
Arabidopsis thaliana MYB37	AJ131517.1	67.9	7e-08
Arabidopsis thaliana DNA for M1 gene	X90379.1	67.9	7e-08
Arabidopsis thaliana chromosome 2	AC005700.3	67.9	7e-08
Antirrhinum majus MYB transcription factor	AY661654.1	65.9	3e-07
P. hybrida myb Ph3 gene	Z13998.1	65.9	3e-07
Medicago truncatula clone mth2-10p1	AC136955.20	65.9	3e-07
Medicago truncatula clone mth2-116k17	AC149493.7	63.9	1e-06
Medicago truncatula clone mth2-23b18	AC122171.21	63.9	1e-06
Medicago truncatula clone mth2-11l3	AC122161.26	63.9	1e-06
Arabidopsis lyrata subsp. *kamchati*	AY387682.1	58.0	7e-05
Arabidopsis lyrata subsp. *kamchati*	AY387681.1	58.0	7e-05

Arabidopsis halleri subsp. *gemmife*	AY387678.1	58.0	7e-05
Medicago truncatula chromosome 7B	AC157894.3	58.0	7e-05
Arabidopsis thaliana R2R3-MYB	AF495524.1	58.0	7e-05
Arabidopsis lyrata Karhuma	AF263721.1	58.0	7e-05
Arabidopsis lyrata Plech glab	AF263720.1	58.0	7e-05
Arabidopsis thaliana gl1-65	AF263719.1	58.0	7e-05
Arabidopsis thaliana gl1-3	AF263718.1	58.0	7e-05
Arabidopsis thaliana Mir glab	AF263717.1	58.0	7e-05
Gossypium hirsutum myb transcription factor	DQ118243.1	58.0	7e-05

Table 3. Myb-related transcriptional factor and their biological function in plant species

Name of gene	*Species*	*Biological function*
CCA1	*Arabidopsis thaliana*	Phytochrome and circadian regulation
LHY	*Arabidopsis thaliana*	Circadian clock regulation, flowering
CPC1	*Arabidopsis thaliana*	Epidermal cell differentiation, root hairs
AtMYBGL1	*Arabidopsis thaliana*	Trichome development
AtMYB13	*Arabidopsis thaliana*	Shoot morphogenesis
AtMYB103	*Arabidopsis thaliana*	Expressed in developing anthers
AtMYB2	*Arabidopsis thaliana*	Dehydration and ABA regulation
ATR1	*Arabidopsis thaliana*	Tryptophan biosynthesis
AtCDC5	*Arabidopsis thaliana*	Cell cycle regulation
AmMYB305, 340	*Antirrhinum majus*	Anthocyanin and flavonol
AmMYB308, 330	*Antirrhinum majus*	Phenolic acid
AmMYBMIXTA	*Antirrhinum majus*	Conical cell development
AmMYBPHAN	*Antirrhinum majus*	Dorsoventral determination and growth
Cpm5, Cpm7, Cpm10	*Craterostigma plantagineum*	Dehydration and ABA response
CotMYBA	*Gossypium hirsutum*	Trichome development
GAMYB	*Hordeum vulgare*	Gibberellin response
NtMYB1	*Nicotiana tabacum*	TMV, SA-inducible
MSA-binding proteins	*Nicotiana tabacum*	Regulation of B-type cyclin genes
BPF1	*Petroselinum crispum*	Telomeric DNA binding protein
PcMYB1	*Petroselinum crispum*	Light-dependent activation
PhMYBAN2	*Petunia hybrida*	Anthocyanin
PhMYB1	*Petunia hybrida*	Conical cell development
PsMYB26	*Pisum sativum*	Phenylpropanoid regulation
ZmMYB1	*Zea mays*	Anthocyanin
ZmMYB38	*Zea mays*	Inhibition of C1-mediated activation
ZmMYBP	*Zea mays*	Phlobaphene

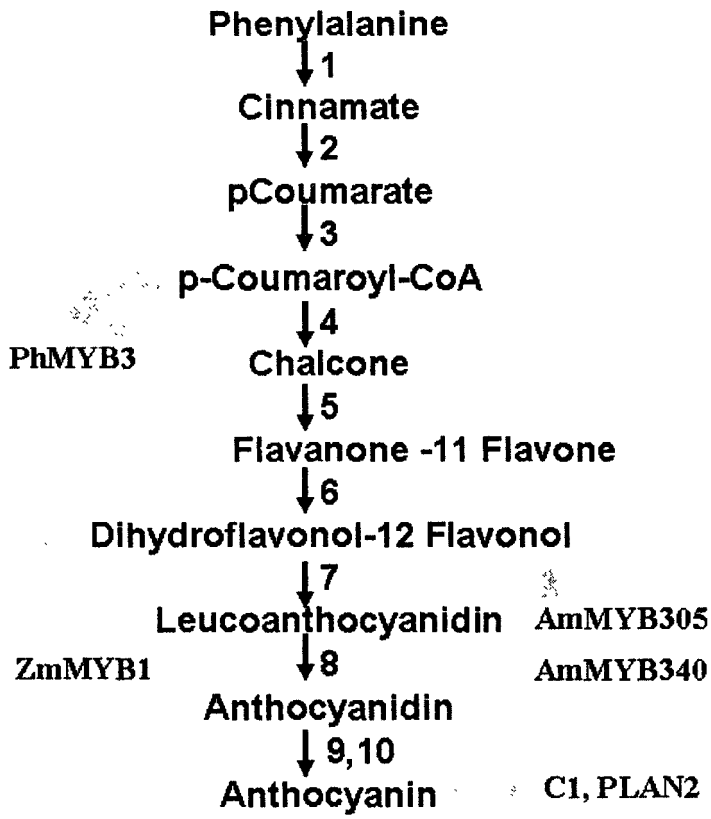

Figure 1. MYB-related transcription factors involved in controlling phenylpropanoid metabolism starts from phenylalanine. MYB-related proteins known to control expression of the subsets of genes encoding enzymes involved in these steps are shown at the end of each pathway. AmMYB305 and AmMYB340 have been shown to activate steps 1, 5, and 6. ZmMYB1 has been shown to activate step 6. PhMYB3 has been shown to activate step 3. C1 and PLAN2 have been shown to activate steps 9 and 10

transcriptions. In response to environmental stimuli, such as light, salt stress or the plant hormones, gibberellic acid and abscisic acid, post-translational control can operate through different mechanisms, including cellular redox potential, phosphorylation, and protein-protein interactions [26-28]. MYB proteins also interact with other transcriptional regulators. Such interactions are widespread for c-MYB, and some are believed to involve interactions with a negative regulatory domain in the C-terminus of the protein that contains a leucine zipper motif [21,28]. No similar domain has yet been described in any plant MYB protein.

To understand the molecular mechanisms of cross-talk between biotic and abiotic stress signaling pathways, we overview the novel functions of MYB transcription factors in stress tolerance and transcriptional control of plant development including: (1) Myb controls cell shape in plant. (2) Myb is response to hormones, (3) Myb is involved in phosphate starvation

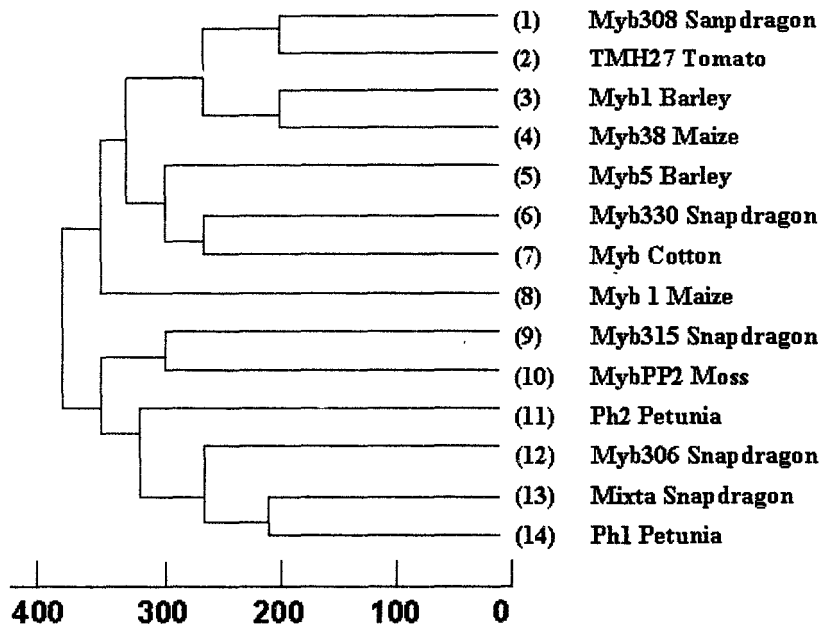

Fig. 2. A neighbor-joining phylogenetic tree of the complete sequence of 14 Myb proteins. Distances are shown as the p-distance multiplied by 1,000. Accession numbers and genus species nomenclature for the taxa are as follows: (1) JQ0960-Antirrhinum majus, (2) 1167486-Lycopersicon esculentum, (3) X70877-Hordeum vulgare, (4) P20025-Zea mays, (5) X70879-Hordeum vulgare, (6) JQ0957-Antirrhinum majus, (7) L04497-Gossypium hirsutum, (8) P20024-Zea mays, (9) JQ0961-Antirrhinum majus, (10)P80073-Physcomitrella patens, (11) Z13997-Petunia hybrida, (12) JQ0956-Antirrhinum majus, (13) X79108-Antirrhinum majus, (14) Z13996-Petunia hybrida.

signaling, (4) Myb regulates phenylpropanoid and lignin biosynthesis, (5) Myb relates to polyphyletic origin and (6) Myb controls the formation of lateral meristems. We intend to provide a genomic perspective on Myb transcription factors with an overview of the transcription-factor-gene content of the *Arabidopsis* genome. We have focused mainly on results from *Arabidopsis* and discussed particular challenges to progress in understanding these genes that have been illustrated by recent studies [13,14]. We consider new results from characterizing the regulatory networks that are formed by transcription factors because more general reviews on the concepts, methodologies and prospects of plant genomics, and on plant transcription factors have been published recently [13,19].

Myb Controls Cell Shape in Plants

The well-established role for plant MYB genes is in the control of cell shape where the *MIXTA* gene of *Antirrhinum* and the orthologous PhMyB1 gene from *Petunia* have been shown to be essential for developing the conical form of petal epidermal cells [13,14] and the *CL1 gene* of *Arabidopsis* has been shown to be essential for the differentiation of hair cells (trichomes) in

some parts of the leaf and in the stem [13]. In maize, another MYB protein, ZmMYE51 can activate one of the structural genes required for anthocyanin biosynthesis, but not the entire pathway [18], while yet another, ZmMyB38, inhibits Cl-mediated activation. Overexpression of *MIXTA* in transgenic tobacco results in trichome formation on petals, suggesting that conical petal cells might be trichoblasts arrested at an early stage in trichome formation. GLI is required for an initial expansion in the size of the cell that develops into the trichome, and it acts upstream of a number of other genes, mutation of which gives rise to cellular outgrowths that do not develop into full, branched trichomes [13,18]. The conical cells produced by the action of the *MIXTA* gene of *Antirrhinum* resemble the limited outgrowths produced in *Arabidopsis g12 mutants* where trichome formation is aborted. Perhaps the initial stages of trichome formation regulated by GLI are similar to those regulated by MIXTA [13]. Two MYB proteins from fungi, the CDCS gene product from *Schizosaccharomyces pombe* and the *FLBD gene* product from *Aspergillus nidulans* can also control aspects of cell shape [13,18]. These similarities in the cellular mode of action of such diverse MYB proteins require understanding of the specific biochemical processes they activate [13,18].

Myb is Response to Hormones

A more-recently defined role for plant MYB proteins is in hormonal responses during seed development and germination [18]. A barley MYB protein (GAhtYB) whose expression is induced by gibberellic acid (GA) has been shown to activate expression of a gene encoding a α-amylase that is synthesized in barley aleurone upon germination for the mobilization of starch in the endosperm [13,18]. Expression of GAMYB is induced by treatment of aleurone layers with GA and expression of the α-amylase gene is induced subsequently. There is a suggestion that other GA-inducible genes can also respond to activation by hWB proteins during seed germination because MYB-like motifs from other GA responsive gene promoters have been shown to direct reporter gene expression in response to GA [13,26]. Treatment with another plant hormone, abscisic acid (ABA), induces expression of ArMYE in *Arabidapsis*, a Myb gene that is also induced in response to dehydration or salt stress. In maize, expression of the *Cl* gene is ABA-responsive, where it is involved in the formation of anthocyanin. AtMYB2 might be responsible for activating expression of some drought-responsive genes because binding by AtMYB2 to the promoter region of a drought- or salt stress-induced gene, *rd22,* has been demonstrated [13,18,27]. The *rd22* gene promoter also contains MYC-recognition sequences suggesting that AtMYB2 can interact with a bHLH protein to induce gene transcription in response to dehydration or salt stress [29,30].

Different types of MYB protein might then have evolved as a result of duplication or triplication of the basic repeat unit [13,18]. It has been proposed that evolution has occurred mostly through modification of regulation of common structural genes, and the separation between different groups of eukaryotes might be accompanied by the differential use of the transcriptional factor classes [31-33]. This does not, in itself, explain why plants have made such extensive use of MYB proteins, and it might well be that MYB genes have been duplicated and their functions expanded in conjunction with the development of novel functions in higher plants [13,18]. Although fungi and bryophytes contain MYBs with two repeats, it is the size of the R2R3-type MYI3 gene family in higher plants that is particularly remarkable [26]. In one lower plant, the MIT3 protein family has, in fact, been estimated to be small, with only two to

three gene members. YYB gene function might have diversified in parallel to increasing complexity in developmental and metabolic pathways as, for example in phenylpropanoid metabolism and also in transcriptional responses to hormones, such as gibberellic acid and abscisic acid, which are specialized plant signaling molecules [13,18]. Plants appear to have used R2R3-type MYB transcription factors selectively to control their specialized physiological functions [13]. However, vertebrates have developed only one small group of MYB proteins to control cellular proliferation and differentiation [13].

Myb is Involved in Phosphate Starvation Signaling

Plants have evolved a number of adaptive responses to cope with growth in conditions of limited phosphate supply involving biochemical, metabolic and developmental changes [34-36]. Rubio *et al.* [37] reported an EMS-mutagenized M2 population of an *Arabidopsis thaliana* transgenic line harboring a reporter gene specifically responsive to phosphate starvation (*AtIPS1-GUS*), and screened for mutants altered in phosphate starvation regulation. One of the mutants, *phr1* (*phosphate starvation response 1*), displayed reduced response of *AtIPS1-GUS* to phosphate starvation, and also had a broad range of phosphate starvation responses impaired, including the responsiveness of various other phosphate starvation-induced genes and metabolic responses, such as the increase in anthocyanin accumulation [37]. *PHR1* was positionally cloned and shown be related to the *PHOSPHORUS STARVATION RESPONSE 1* (*PSR1*) gene from *Chlamydomonas reinhardtii*. A GFP-PHR1 protein fusion was localized in the nucleus independently of phosphate status, as is the case for PSR1. *PHR1* is expressed in Pi sufficient conditions and, in contrast to *PSR1*, is only weakly responsive to phosphate starvation [37]. PHR1, PSR1, and other members of the protein family share a MYB domain and a predicted coiled-coil (CC) domain, defining a subtype within the MYB superfamily, the MYB–CC family [13,18]. Therefore, PHR1 was found to bind as a dimer to an imperfect palindromic sequence. PHR1-binding sequences are present in the promoter of phosphate starvation-responsive structural genes, indicating that this protein acts downstream in the phosphate starvation signaling pathway [37].

Myb Regulates Phenylpropanoid and Lignin Biosynthesis

MYB-related transcription factors are known to regulate different branches of flavonoid metabolism in plants and are believed to play wider roles in the regulation of phenylpropanoid metabolism in general [13,18]. Tamagnone *et al.* [38] demonstrate that overexpression of two *MYB* genes from *Antirrhinum* represses phenolic acid metabolism and lignin biosynthesis in transgenic tobacco plants. The inhibition of this branch of phenylpropanoid metabolism appears to be specific to AmMYB308 and AmMYB330, suggesting that they recognize their normal target genes in these transgenic plants [38]. Experiments with yeast indicate that AmMYB308 can act as a very weak transcriptional activator so that overexpression may competitively inhibit the activity of stronger activators recognizing the same target motifs [13]. The effects of the transcription factors on inhibition of phenolic acid metabolism resulted in complex modifications of the growth and development of the transgenic plants [39-41]. The inhibition of monolignol production resulted in plants with at least 17% less lignin in their vascular tissue. This reduction is of importance when designing strategies for the genetic modification of woody crops [38].

Myb Relates to Polyphyletic Origin

The Myb family of proteins is a group of functionally diverse transcriptional activators found in both plants and animals that is characterized by a conserved DNA-binding domain of approximately 50 amino acids [13,18,19]. Phylogenetic analyses of amino acid sequences of this family of proteins portray very disparate evolutionary histories in plants and animals [42]. Animal Myb proteins have diverged from a common ancestor, while plants appear related only within the DNA-binding domain [13]. Results imply a pattern of modular evolution of the Myb proteins centering on the possession of a helix-turn-helix motif. Based on this, it is suggested that Myb proteins are a polyphyletic group related only by a 'Myb-box' DNA-binding motif [13,42]. However, other transcription factors such as MADS-box protein is involved in the control of floral architecture [43]. Zinc fingers and basic leucine zipper transcription factors are regulating interfascicular fiber differentiation or being response to abscisic acid signaling in *Arabidopsis* [44-46].

Myb Controls the Formation of Lateral Meristems

The multitude of forms observed in flowering plants is largely because of their ability to establish new axes of growth during postembryonic development [13,47,48]. This process is initiated by the formation of secondary meristems that develop into vegetative or reproductive branches. In the *blind* and *torosa* mutants of tomato, initiation of lateral meristems is blocked during shoot and inflorescence development, leading to a strong reduction in the number of lateral axes [48-50]. Schmitz *et al.* [49] reported that *blind* and *torosa* are allelic. The *Blind* gene has been isolated by positional cloning and it was found that the mutant phenotype is caused by a loss of function of an R2R3 class *Myb* gene. RNA interference-induced *blind* phenocopies confirmed the identity of the isolated gene. Double mutant analysis shows that *Blind* acts in a novel pathway different from the one to which the previously identified *Lateral suppressor* gene belongs [49]. The findings reported add a new class of transcription factors to the group of genes controlling lateral meristem initiation and reveal a previously uncharacterized function of R2R3 *Myb* genes [13,49].

Conclusions

Transcription factors play an important role in plant stress tolerance, phytohormone signaling, and organ development [40,51-53]. A holistic view of the regulation of transcription will ultimately emerge from the genomic analyses of transcription factors [13]. For *Arabidopsis*, as for any other organism, progress toward this goal will begin with comprehensive analyses of the genome sequence to identify transcription-factor genes as well as the sequences on which they act and the genes that they regulate [13]. Extensive DNA microarray experiments, the genome wide mapping of protein-protein interactions, and the comprehensive phenotypic characterization of mutants for all transcription-factor genes will then be necessary [13,18]. The plethora of data generated must be correlated and integrated. Finally, the practical application of transcription factor in agricultural and environmental biotechnology will be depend on the genetic engineering of these genes in economically important plant species [54-56]. Our recently established transformation system will speed the application of these transcription factors in pine [57].

Acknowledgements

The authors are grateful to undergraduate research assistants Karina Hesterberg, Ruba Sarsour, Adaeze Okoye, Hubert Chia, and Zalak Daftary, for their work in isolating mature embryos from seeds for callus induction. This work was supported by the East Carolina Christmas Tree Program.

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5 Cell Death and Reactive Oxygen Species During Accelerated Ageing of Soybean (Glycine max L.) Axes

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Introduction

In commerce, seeds are subjected to varying degrees of water and temperature stresses during development, maturation, harvest, and storage in seed production and distribution, and then during imbibition and germination following sowing. Only those seeds which have high vigor best able to cope with these conditions and are capable of rapid germination and establishment of healthy seedlings, which are essential for efficient crop production. Seed deterioration leads to reductions in seed quality, performance, and seedling establishment. While it is difficult to quantify the economic loss caused by poor seed performance, one estimate has been suggested that it is 500 million dollars annually just for purchased seed. Thus it is important that a fundamental understanding of the processes of seed deterioration be gained [18].

Seed deterioration is due in part to the reason of membrane lipid peroxidation and leakiness caused by reactive oxygen species (ROS) attacking [3, 29], including surperoxide radical ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$) and other organic radical [24]. At the cellular level, the excess production of ROS causes cell death [30].

Cell death can be divided into two types, necrotic and programmed cell death (PCD), both can be induced by the ROS [11]. Changes in protein structure and nucleic acid damage can also attribute to ROS attacking [18], therefore chromosomal mutation accumulates and the onset of mitosis for cell division and germination delay. ROS scavenging enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and glutathion reductase (GR) participate metabolism of the ROS, and they inhibit and reduce the damage [15, 18, 31].

The processes by which stored seeds die has received considerable attention in the literature [31], these processes mainly included decline in fatty acid content [22, 33], decrease in non-enzymatic antioxidant level [3, 24] and ROS scavenging enzyme activity [2, 26, 28] and increase

in malondiadlehyde level [2, 28], to our knowledge, to data did not implicate in cell death, production of ROS, and particularly in relationships among seed vigor, cell death, and production and scavenging of ROS during accelerated ageing of seeds.

The loss in seed viability during storage is a gradually process. Water content of the seeds and storage temperature are major factors in determining seed viability during storage [5]. Accelerated ageing of seeds, seed lot was exposed to high temperature and high relative humidity (RH), leads to the loss of vigor and eventually viability, and is an excellent method to determine the vigor changes during seed storage. In this paper, soybean seeds were used as experimental materials, accelerated ageing of seeds was used to simulate or to substitute the natural ageing, relationships among seed vigor, cell death, and production and scavenging of ROS during accelerated ageing were studied, and a model to explain above-mentioned relationships was suggested based on our research results.

Materials and Methods

Plant Material

Current harvested soybean (*Glycine max* L. cv. Aijiaozao) seeds (purchased from Institute of Oil Crops Research, Chinese Academy of Agriculture Sciences) were placed into a nylon mesh bag, and then suspended in a closed desiccator (Φ = 22 cm), and were subjected to accelerated ageing at 40°C and 100% RH for 0, 5, 10, 15 and 20 d, respectively.

Determination of Water Content

Water content of seeds was determined gravimetrically (103°C for 18 h). Thirty seeds were sampled for each determination. Water content of seeds is expressed on a dry mass basis [g H_2O (g dry mass)$^{-1}$; g/g].

Germination Test

Batches of 50 seeds were germinated on two filter paper and 15 ml deionised water in Petri dishes (Φ = 12 cm) at 20°C in the dark for 5 days. Seeds showing radicle emergence were scored as germinated. Fresh weight of seedlings produced by germinating seeds does not include cotyledons.

Viability Stain

The cross and near-median longitudinal sections of soybean radicle (approximately 3 mm thick) were stained in 0.1% (w/v) Evans blue for 2 min by the method of Levine *et al.* [16]. Stained sections were rinsed in deionised water for 30 min, and photographed with Kodak MAX 400 film on the Olympus stereomicroscope.

Measurement of Respiratory Rate

Respiratory rate of single seed was measured according to the method of Xue [32]. Oxygen uptake by the whole seed was measured at 25°C using a SKW-2 micro pressure detector. Volume of the reaction bottle was determined by the weight of mercury (Hg) that contained.

Determination of Superoxide Radical and Hydrogen Peroxide

Superoxide radical was measured as described by Elstner and Heupel [9] by monitoring the nitrite formation from hydroxylamine in the presence of superoxide radical, modified as follows. Axes (about 0.3 g) were homogenized in 6 ml of ice-cold 50 mM sodium phosphate buffer (pH 7.8) at 4°C, and the brei was centrifuged at 12,000 g for 10 min. The supernatant was used for determination of superoxide radical. The reaction mixture contained 0.9 ml of 50 mM phosphate buffer (pH 7.8), 0.1 ml of 10 mM hydroxylamine hydrochloride, and 1 ml of the supernatant was incubated at 25°C for 20 min, and then 0.5 ml of 17 mM sulfanilamide and 0.5 ml of 7.mM napathylamine were added to the reaction mixture. After incubation at 25°C for 20 min, the absorption in the aqueous solution was read at 530 nm. A standard curve with nitrite was used to calculate the production rate of superoxide radical from the chemical reaction of superoxide radical and hydroxylamine.

The content of hydrogen peroxide was measured by monitoring the absorption of titanium-peroxide complex at 410 nm according to the method of MacNevin and Uron [17] and Partterson *et al.* [21], modified as follows. Axes (about 0.3 g) were homogenized in 6 ml of 5% (w/v) trichloroacetic acid, and then centrifuged at 12000×g for 10 min. After 1 ml supernatant and 9 ml of 0.2% (w/v) titanium tetrachloride hydrochloride solution were mixed, and the absorbance of reaction solution was measured at 410 nm, and using H_2O_2 as a standard.

Assay of SOD

Axes (about 0.1 g) were ground with a mortar and pestle at 4°C in an extraction mixture composed of 50 mM phosphate buffer (pH 7.0), 1.0 mM EDTA, 0.05% (v/v) Triton X-100, 2% (w/v) PVPP and 1 mM ascorbic acid. The homogenate was centrifuged at 16,000 g for 15 min, after which the supernatant was transferred to a new tube and kept at -20°C.

SOD (EC 1.15.1.1) activity assay was based on the method of Beauchamp and Fridovich [4], who measure inhibition of the photochemial reduction of NBT at 560 nm, modified as follows. The 1 ml reaction mixture contained 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 75 mM NBT, 16.7 mM riboflavin and enzyme extract (ca 50 μg protein). Riboflavin was added last and the reaction was initiated by placing the tubes under two 9-W fluorescent lamps. The reaction was terminated after 15 min by removal from the light source. An illuminated blank without protein gave the maximum reduction of NBT, and therefore, the maximum absorbance at 560 nm. SOD activity (mean of five replicates) is presented as absorbance of sample divided by absorbance of blank, giving the % of inhibition. In this assay, 1 unit of SOD is defined as the amount required to inhibit the photoreduction of NBT by 50%. The specific activity of SOD was expressed as unit mg^{-1} protein.

Assays of APX, CAT and GR

Axes (about 0.2 g) were ground with a mortar and pestle at 4°C in 5 ml of 50 mM Tris-HCl (pH 7.0), containing 20% (v/v) glycerol, 1 mM AsA, 1 mM dithiothreitol, 1 mM EDTA, 1 mM GSH, 5 mM $MgCl_2$ and 1% (w/v) PVPP. After two centrifugation steps (12,000 g for 6 min and

26,900 g for 16 min, respectively), the supernatant was stored at -20°C for later determinations of enzyme activities of APX, CAT and GR.

APX (EC 1.11.1.7) was assayed as the decrease in absorbance at 290 nm due to AsA oxidation, by the method of Nakano and Asada [20]. The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 1 mM sodium ascobate, 2.5 mM H_2O_2 and enzyme source (ca 50 μg protein) in a final volume of 1 ml at 25°C.

CAT (EC 1.11.1.6) activity was determined by directly measuring the decomposition of H_2O_2 at 240 nm as described by Aebi [1], in 50 mM potassium phosphate (pH 7.0), containing 10 mM H_2O_2 and enzyme source (ca 50 μg protein) in a final volume of 1 ml at 25°C.

GR (EC 1.6.4.2) was determined as the decrease in absorbance at 340 nm due to the oxidation of NADPH, according to Halliwell and Foyer [13], in 50 mM Tris-HCl buffer (pH 7.5), containing 5 mM $MgCl_2$, 0.5 mM GSSG, 0.2 mM NADPH and enzyme extract (ca 100 μg protein) in a final volume of 1 ml at 25°C.

Lipid Peroxidation Product

Lipid peroxidation products were determined as the concentration of TBA-reactive substances, equated with MDA, by the method of Hodge *et al.* [14]. Content of MDA was calculated according to C_{MDA} (nmol L^{-1}) $=6.45\times[(A_{532}-A_{600})-0.0571\times(A_{450}-A_{600})]$, and was expressed as nmol mg^{-1} protein.

Protein Assay

Protein was measured following the procedure of Bradford [6], using BSA as a standard.

Statistical Analysis

The data of changes in water content, final germination percentages, fresh weight of seedling during accelerated ageing of soybean seeds were analysed using a one way ANOVA model from the SPSS 11.0 package for Windows (SPSS Inc.).

Results

Changes in Water Content and Germination of Seeds

Water content of dried soybean seeds was originally 0.102±0.002 g/g, and increased rapidly during the early stage of accelerated ageing (40°C, 100% RH), and then slowly increased until 15 d of accelerated ageing, and then rapidly increased (Figure 1a; Table 1).

The final germination percentage of soybean seeds decreased with accelerated ageing, and decreased to zero till 20 d of accelerated ageing (Figure 1a; Table 1); the accelerated ageing time when final seed germination was decreased to 50% (T_{50}) was about 10.5 d. Seed vigor, as measured by fresh weight of seedling produced by germinating seeds at 5 d of imbibition (Figure 1a; Table 1) and germination rate of seeds (Figure 1b), also decreased with increasing accelerated ageing time.

Table 1. Changes in water content, germination and fresh weight of seedling during accelerated ageing of soybean seeds. All values are means ± SD of three replicates of 50 seeds each and statistical results of a one-way ANOVA. d.f., degrees of freedom; MS, mean squares

Accelerated ageing time (d)	*0*	*5*	*10*	*15*	*20*	*d.f.*	*MS*	*F- ratios*	*P-value*
Moisture content (g H_2O/g DW)	0.102±0.002	0.325±0.007	0.335±0.017	0.365±0.013	0.481±0.013	4	0.057	198.100	0.000
Germination (%)	95.0±0.000	85.0±0.000	53.33±5.210	30.0±5.000	0.0±0.000	4	4594.170	145.080	0.000
Fresh weight of seedling (g/plant)	0.241±0.063	0.179±0.037	0.167±0.040	0.150±0.012	0.0±0.000	4	0.240	7.844	0.040

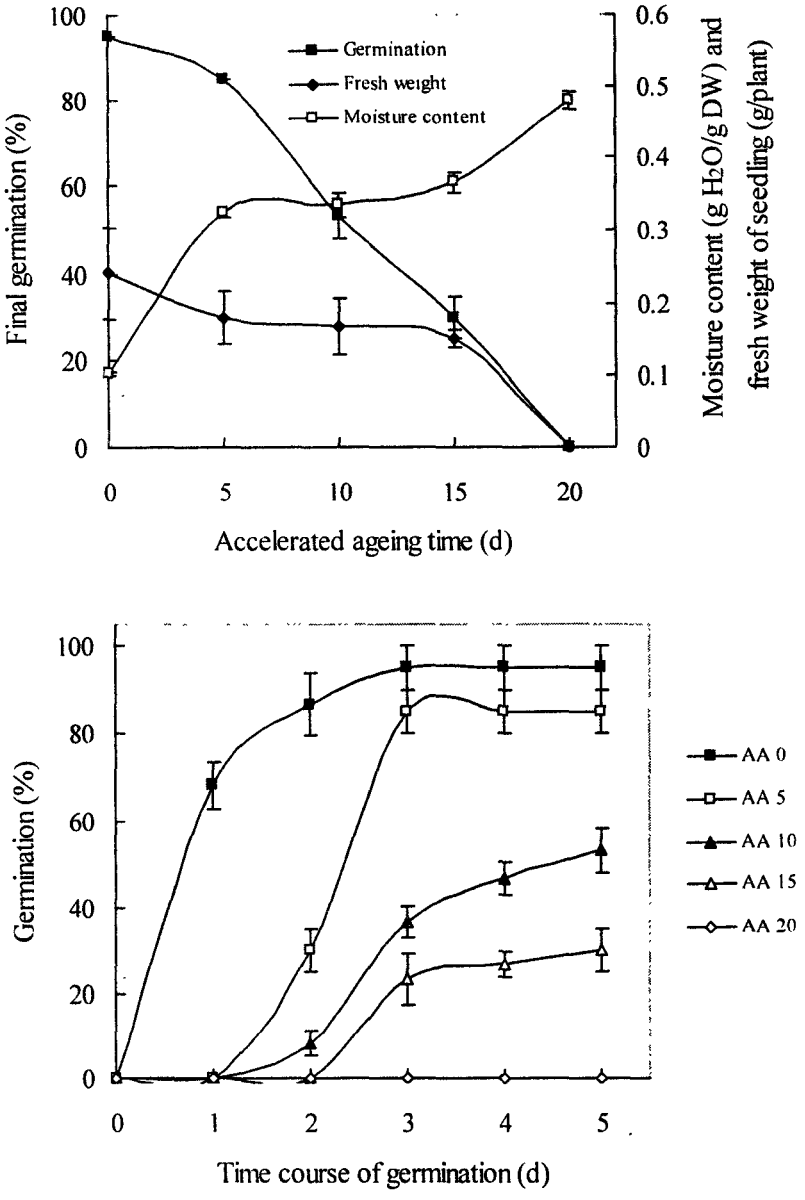

Figure 1. Changes in water content, final germination, fresh weight of seedling (a), and germination rate during accelerated ageing of Glycine max seeds. After seeds were accelerated ageing at 40°C and 100% RH for indicated time, water content of seeds were determined, and seeds were germinated at 20°C in the dark for indicated time. Seeds showing radicle emergence were scored as germinated. Fresh weight of seedlings produced by germinating seeds does not include cotyledons. All values are means ± SD of three replicates of 50 seeds each. AA, accelerated ageing.

Detection of Cell Death

The viability of soybean radicle cells during accelerated ageing was examined by staining fresh hand sections with Evans blue, a dye that is excluded from living cells with intact plasma membranes, thereby staining was only in the cytoplasm of nonviable cells. Little Evans blue staining of cells in the cross and near-median longitudinal sections of non-aged soybean radicle was observed (Plate Ia, b). The radicle cells, especially in meristematic cells stained by Evans blue, gradually increased with increasing accelerated ageing time (Plate Ic-j), indicating loss of cell viability.

Respiratory Rate

Respiration rate of seed markedly increased with accelerated ageing, peaked on the 10 d of accelerated ageing, and then decreased (Figure 2). Respiration rate (1328 ± 111 μl O_2 g^{-1} DW h^{-1}) of seed accelerated aged for 20 d was still higher than that of non-aged seed (753 ± 109 μl O_2 g^{-1} DW h^{-1}), though final germination of seed became zero, (Figure 2).

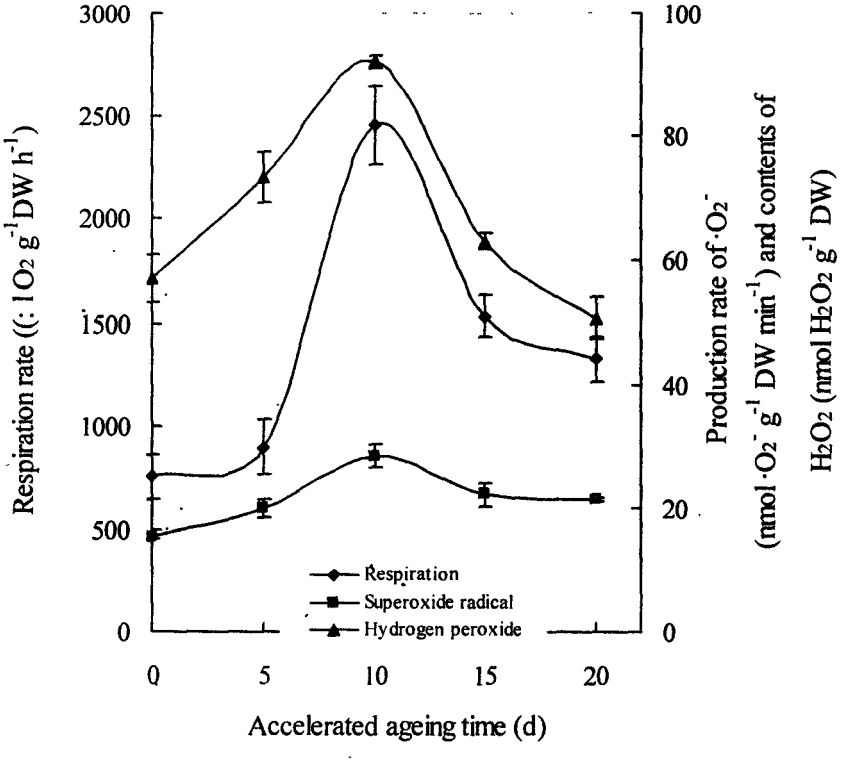

Figure 2. Changes in respiration, superoxide radical, and hydrogen peroxide during accelerated ageing of Glycine max seeds. Accelerated ageing of seed, determination of superoxide radical and hydrogen peroxide were carried out as described in Materials and Methods. All values are means ± SD of five replicates.

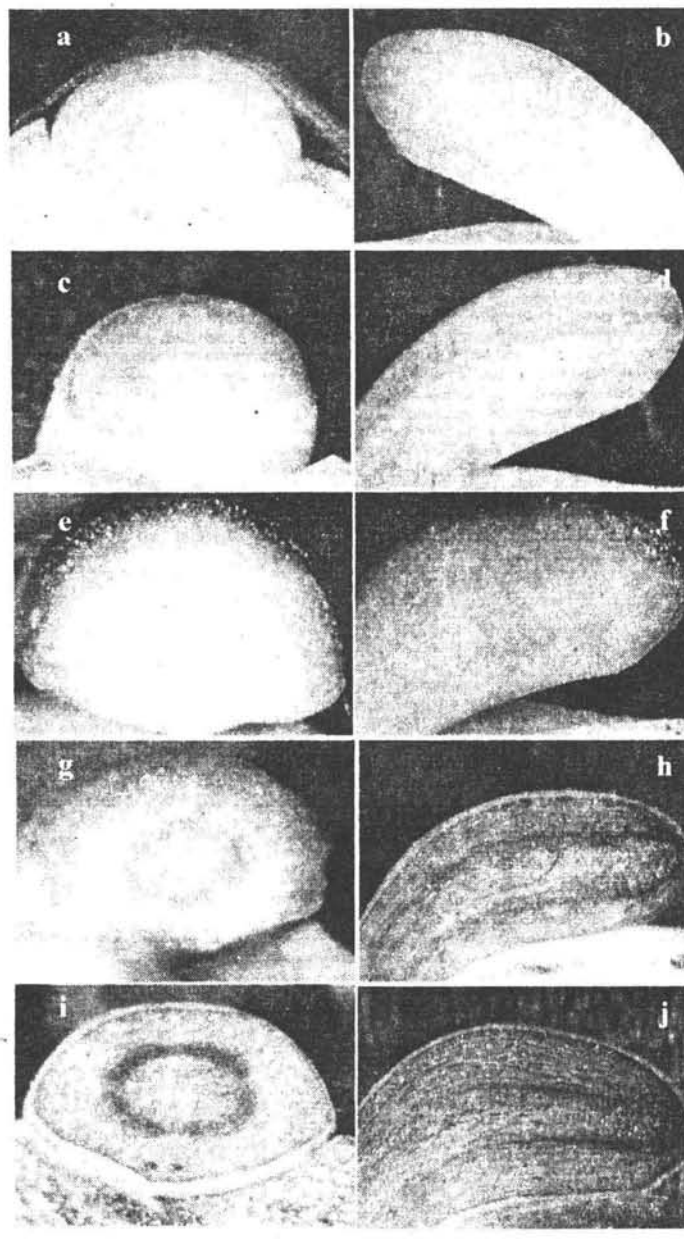

Plate I. Progress of radicle cell death during accelerated ageing of Glycine max seeds, as indicated by Evans blue staining. Accelerated ageing of soybean seeds and viability staining of cross and longitudinal section of soybean radicle were carried out as described in Materials and methods. Accelerated ageing time shown are: a and b, 0 d; c and d, 5 d; e and f, 10 d; g and h, 15 d; I and j, 20 d. a, c, e, g and i indicate cross sections of soybean radicle, and b, d, f, h and j, longitudinal sections.

Changes in Superoxide Radical and Hydrogen Peroxide

After soybean seeds were accelerated aged for different time, and axes were immediately excised. $\cdot O_2^-$ production rate and H_2O_2 content of soybean axes increased with accelerated ageing, peaked at the 10 d of accelerated ageing, and then decreased (Figure 2). For example, $\cdot O_2^-$ production rate and H_2O_2 content of the axes accelerated for 10 d increased by 85% and 61%, respectively, compared to non-aged axes (control) (Figure 2).

Activities of SOD, CAT, APX and GR

Activities of SOD and APX of soybean axes decreased slowly at initial stage of accelerated ageing, and then increased at 15 d, and final decreased at 20 d; activities of SOD and APX of axes accelerated aged for 20 d decreased by 8% and 57% than those of non-aged axes, respectively, (Figure 3a).

CAT activity of axes gradually decreased with accelerated ageing (Figure 3b). The changes of GR activities of axes were similar to those of SOD and APX during accelerated ageing, but its activity peak was about at 10 d of accelerated ageing (Figure 3b).

MDA Content

MDA content of axes markedly increased with accelerated ageing, and increased by 66% and 60%, respectively, by the 15 d and 20 d of accelerated ageing, compared to non-aged axes (Figure 3c).

Discussion

Under accelerated ageing (40°C, 100% RH), changes in water content of soybean seeds exhibit a three-phase process of water uptake (Figure 1a; Table 1), which could consist of imbibition (I), deteriorative metabolism (II) and passive absorption caused by ambient 100% RH (III).

The final germination percentage (Figure 1a; Table 1) and germination rate (Figure 1b) of seeds, and fresh weight of seedling produced by germinating seeds decreased with increasing accelerated ageing, as found for *Arachis hypogaea* seeds by Song *et al.* [26], for wheat seeds by Guy and Black [12] and for *Beta vulgaris* seeds by Song *et al.* [27]. The symptoms observed during accelerated ageing can be used to characterize the degree of ageing, which varies in the opposite direction as storability. Stability against accelerated ageing has subsequently been recognized as a useful vigor test for some species [23]. The physiological and biochemical changes during rapid deterioration of seeds have been increasingly used as indices of ageing [23].

Viability staining by Evans blue showed that the death of radicle cells, especially meristematic cells of axes gradually increased with accelerated aged time (Plate Ia-j). As determined by tetrazolium staining, deterioration begins with the root and moves through the embryo in both naturally and artificially aged *Triticum aestivum* seeds [7, 8]; and embryonic axes of *A. hypogaea* were the most sensitive seed parts to deterioration [10].

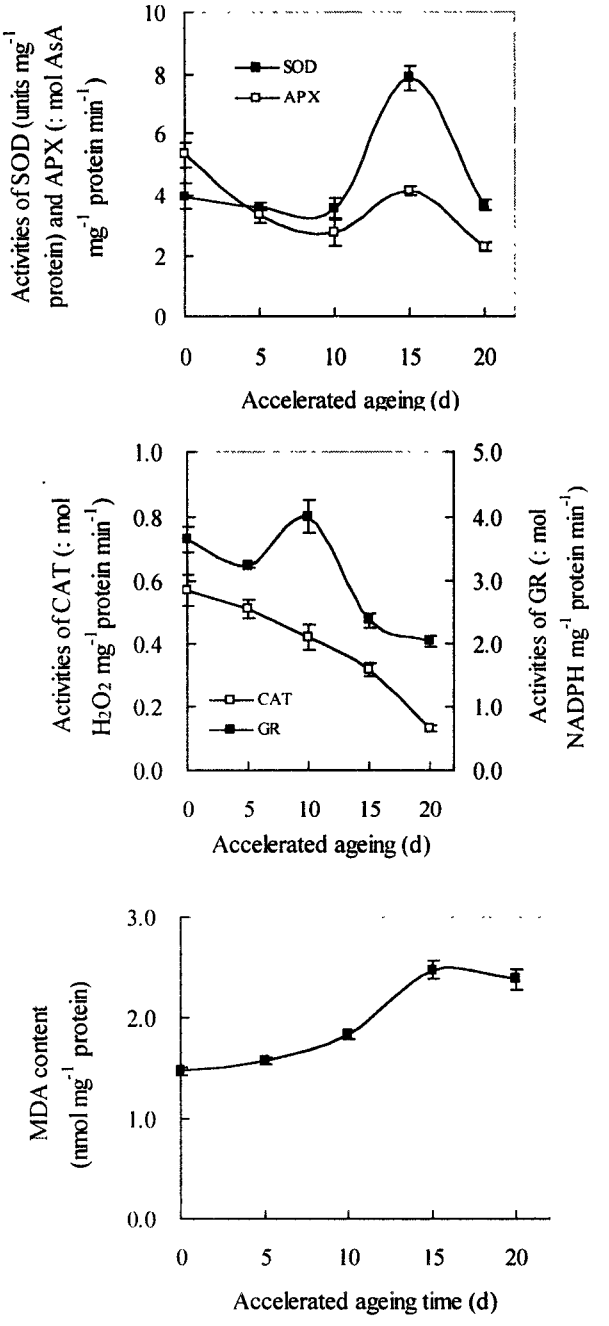

Figure 3. Changes in activities of SOD, APX (a), CAT and GR (b), and MDA content (c) during accelerated ageing of Glycine max seeds. Accelerated ageing of seed, assay of SOD, APX, CAT and GR activities, and determination of MDA content were carried out as described in Materials and Methods. All values are means ± SD of three replicates.

Respiration rate of seed, $\cdot O_2^-$ production rate and H_2O_2 content of axes markedly increased with accelerated ageing, peaked at the 10 d of accelerated ageing, and then decreased (Figure 2). The imbibition of water by the dry seed can resume cell activities at the initial stage of accelerated ageing, increase in seed respiration rate might be due to enhancement of mitochondria activity. Whereas decrease in respiration rate might be that structure and function of mitochondria were damaged by further accelerated ageing. Mitochondria of the root tip cells of ageing-accelerated maize seeds were among the first organelles to show damage [25]. The production of ROS, such as $\cdot O_2^-$, and H_2O_2, is an unavoidable consequence of aerobic metabolism. In plant cell, the mitochondrial electron transport chain is a major site of ROS production [19]. Changes in $\cdot O_2^-$ production rate and H_2O_2 content of axes were similar to those of respiration rate, indicated that increase in respiration rate, especially excessive electrons produced by abnormal oxidative phosphorylation under stress of accelerated ageing would provide electrons for production of ROS via electron leakage. Song *et al.* [27] showed that activities and latencies of cytochrome c oxidase (EC 1.3.9.1) and malate dehydrogenase (EC 1.1.1.37) considerably decreased with accelerated ageing of *Beta vulgaris* seeds.

Activities of SOD, APX and GR of soybean axes decreased at initial stage of accelerated ageing, and then slightly increased (with the exception of SOD activity), and final decreased (Figure 3a,b); CAT activity of axes decreased with accelerated ageing (Figure 3b). MDA is one of main products of lipid peroxidation. MDA content of axes markedly increased with accelerated ageing (Figure 3c). These results were in accordance with the findings for *A. hypogaea* seeds by Song *et al.* [26] and Sung and Jeng [29], for *Helianthus annuus* seeds by Bailly *et al.* [2], who demonstrated that loss of seed viability was associated with a decrease in SOD, CAT and GR, and that accelerated ageing induced accumulation of MDA. McDonald [18] considered that production of ROS which caused lipid peroxidation may be a principal cause of seed deterioration.

Based on experimental results mentioned above, a model can be established to explain the relationships among seed vigor, cell death, production and scavenging of ROS and lipid peroxidation during accelerated ageing (Figure 4). When seeds were subjected to accelerated ageing (40°C, 100% RH), respiration rate was induced rise, ROS production caused by excess electrons was enhanced, the activities of ROS scavenging enzymes decreased, lipid peroxidation increased, finally cells were killed by these events, and seed viability lost (Figure 4).

Acknowledgements

We are grateful to the Knowledge Innovation Project of the Chinese Academy of Sciences (KSCX2-SW-117), Hundreds Talent Program of the Chinese Academy of Sciences, and Natural Science Foundation of Yunnan (2003C0068M) of China for supporting.

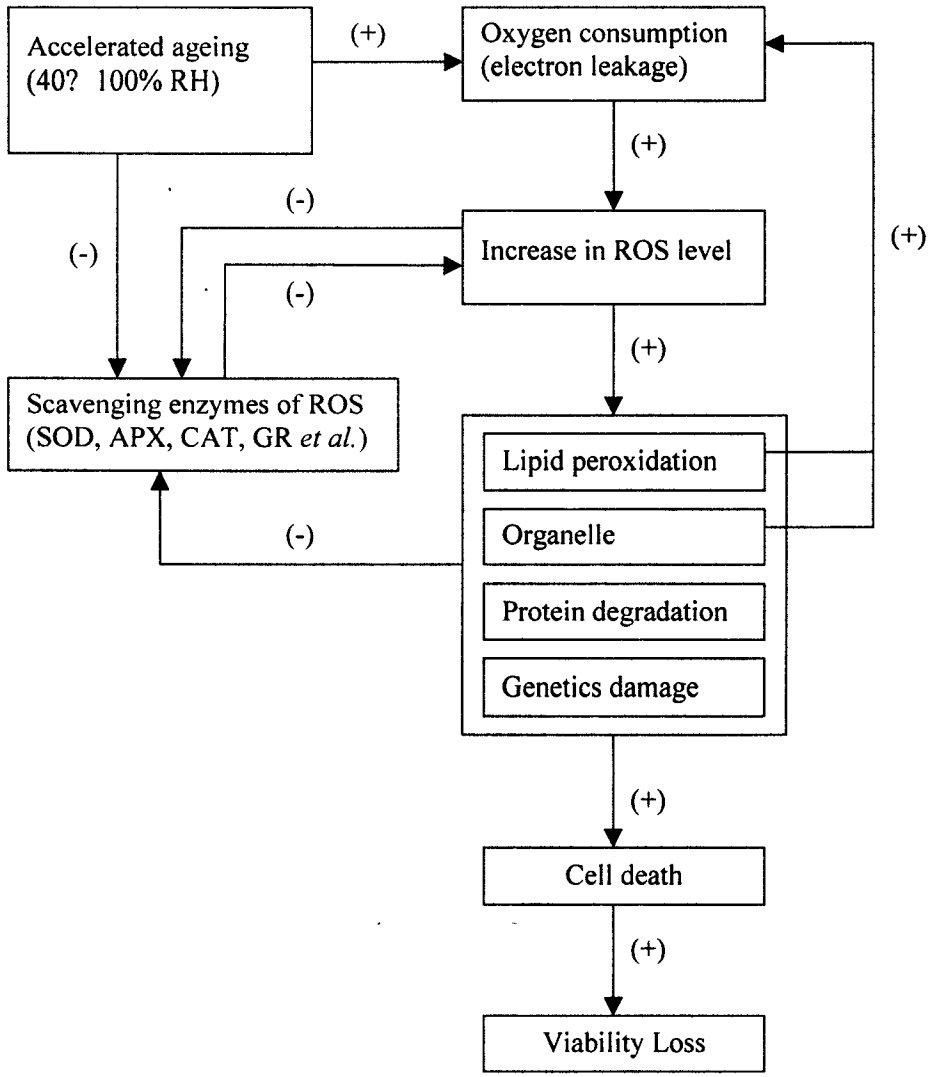

Figure 4. A model to explain the relationships among seed vigor, cell death, production and scavenging of ROS and lipid peroxidation during accelerated ageing. (+) means inducement; (-) means inhibition.

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6 Metabolism of Polyamines and Prospects for Producing Stress-tolerant Plants: An Overview

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Introduction

Polyamines (PAs) like putrescine^{+2} (Put), spermidine^{+3} (Spd), spermine^{+4} (Spm), and cadaverine^{+2} (Cad) are low molecular weight organic polycations displaying a high biological activity. They are universal, multifunctional regulators of physiological processes, exhibiting anti-stress protective effects in particular. Polyamines are present in all compartments of the plant cell, including nucleus, which indicates their participation in diverse fundamental processes in the cell [1-3]. Like hormones, polyamines are involved in the processes of replication, transcription, translation, membrane stabilization, enzyme activity modulation, cell division and elongation, plant growth and development [4-6]. The concentrations of PAs in the plant (10^{-9}–10^{-5} M) are much higher than those of endogenous phytohormones (10^{-13}–10^{-7} M). The total PA concentration and the ratios between individual PAs vary markedly in dependence on plant species, organ, and tissue, and also on the developmental stage. Stress-induced PA accumulation and their protective function against stresses are of special interest [7].

The pathways of PA biosynthesis in higher plants are well studied, most of the genes for enzymes involved are cloned and their regulation has been studied. Nevertheless, in order to understand properly the role of PAs during plant development under normal and stress conditions, it seems of importance to critically analyze and summarize the accumulated experimental data, focusing the compartmentation and enzymology of PA biosynthesis and catabolism, their transmembrane and interorgan transport, and the molecular mechanisms of their protective effects. In this review, we also consider transgenic and mutant plants displaying changed PA metabolism and the usage of specific inhibitors.

Polyamine Biosynthesis

Putrescine

Put is the first member of the family of usual PAs, a precursor of Spd and Spm. Put is synthesized by the two ways: directly from ornithine by ornithine carboxylase (ODC, EC 4.1.1.17) or via several intermediates from arginine with the involvement of arginine decarboxylase (ADC, EC 4.1.1.19) (Figure 1). The product of the latter reaction is agmatine, which is converted by agmatine iminohydrolase (EC 3.5.3.12) into N-carbamoylputrescine and then by aminohydrolase (EC 3.5.1.53) into Put. A possibility of the direct conversion of citrulline into Put by citrulline decarboxylase is also discussed [8]. However, the presence of this enzyme in plants is not yet proven [9].

The formation of Put with the involvement of ADC is usually associated with the plant responses to stress. By the methods of molecular biology, it was shown that ADC was localized in essentially all organs of *Nicotiana tabaccum* L. (flowers, stems, seeds, leaves, and roots) [10,11]. Using immunoenzyme approaches, it was demonstrated that ADC protein was present in the two different compartments: in chloroplasts in the leaves (photosynthesizing organs) and in nuclei in the roots (nonphotosynthesizing organs), which may be related to specific functions of ADC in different cell types.

ADC pathway of Put synthesis does not evidently function in mammals and humans [8,12], although some reports are known about its functioning. Arginine is an essential amino acid. Animals and humans obtain it from plants with food and convert into ornithine by arginase (CE 3.5.3.1), which is used for PA synthesis by ODC. It should be noted that ODC is extremely labile: its half-life in animals does not exceed 60 min.

The analysis of published data shows that Put accumulation and ADC activation occur, as a rule, under unfavorable conditions such as drought, salinity, hyperthermia, potassium and sulfur deficits etc. [7]. A stress-induced activation of the complete pathway of PA biosynthesis with the accumulation of Spd and Spm is observed relatively infrequently [13,14]. Usually, in sensitive plant species, stress induces the selective accumulation of Put [15-18]. In contrast, in tolerant plant species, for example in the salt-tolerant rice cultivar [9] and salt-tolerant tobacco *NrEs-1* strain, salinity induced 10- to 15-fold accumulation of Spd as compared to control cells [20,21]. It is possible to suppose that, in sensitive species, stress suppresses the key enzyme of Spd and Spm biosynthesis, SAM decarboxylase (SAMDC). Therefore, the biosynthesis of these two PAs is retarded, and their precursor Put accumulates actively.

ODC pathway functions more actively in juvenile plants. In fact, an increased arginase activity catalyzing ornithine formation and tightly associated with chromatin [8] was detected in plants only at early developmental stages, whereas its activity was suppressed in adult plants [22,23]. This means that ODC dominates in PA biosynthesis in the period of active cell division at early stages of plant development [11,24] and also in developing fruits due to intense ornithine formation in mitochondria [25,26]. Moreover, it was established that the ornithine pathway of Put biosynthesis prevailed in roots, where mitochondria were the main source of ornithine as well [23,27,28]. It is of importance that the high level of ODC gene expression in roots was characteristic for the period of pollination and during plant defense against bacterial and fungal infections [29-31]. It is of interest that, in pepper (*Capsicum annuum* L.) plants resistant to

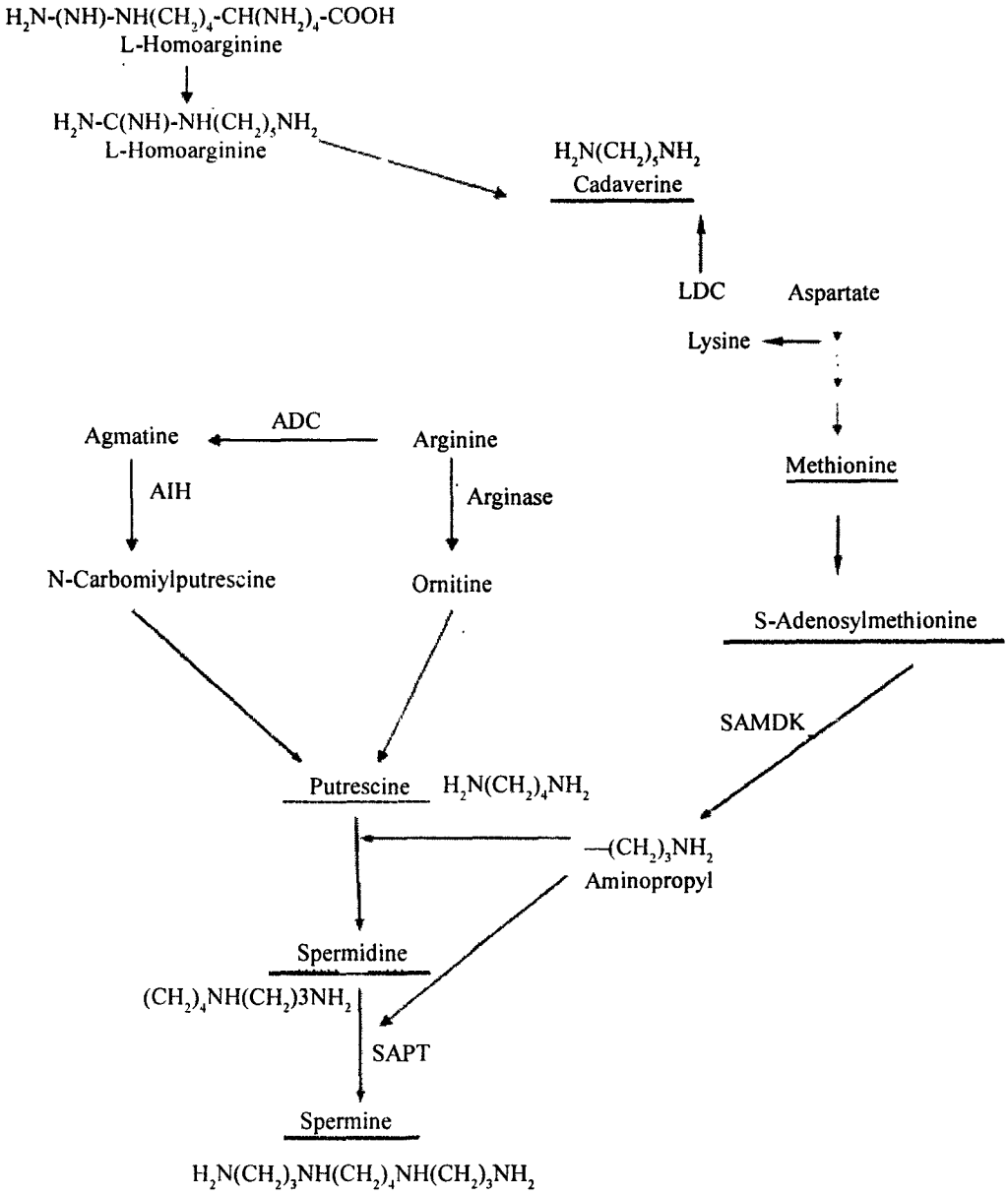

Figure 1. Pathways of biosynthesis of the major plant polyamines (putrescine, spermidine, spermine and cadaverine).

tobacco mosaic virus, three ODC genes were found [31-32], which displayed differential expression in response to pathogen action.

Like in animals, plant ODC is localized in the nucleus [1-3] and is tightly associated with chromatin [8], whereas ADC is localized in thylakoid membranes of chloroplasts [14,28,33].

It was established, for example, that spinach ADC was associated with LHC of photosystem II [33-35]. PAs synthesized in chloroplasts evidently stabilize photosynthetic complexes of thylakoid membranes under stress conditions [33,36,37]. Physical uncoupling and independent functioning of the two pathways of Put biosynthesis [38-39] may indicate a difference in their functions, whereas chloroplast and mitochondrial membranes can serve barriers separating ornithine, the product of catabolism from ornithine used for arginine biosynthesis [8].

Spermidine, Spermine and Unusual Polyamines

Spd and Spm are synthesized by successive attachment of aminopropyl first to Put and then to Spd (Figure 1). These reactions are catalyzed by aminopropyltransferases, Spd synthase (EC 2.5.1.16), and Spm synthase (EC 2.5.1.22), which are also known as Put aminopropyltransferase (PAPT) and Spd aminopropyltransferase (SAPT). Aminopropyl is formed due to decarboxylation of S-adenosylmethionine (SAM) by SAM decarboxylase (SAMDC, EC 4.1.1.50), which has a very short half-life (from 5 to 60 min) and is a rate-limiting enzyme of Spd and Spm biosynthesis. PAPT and SAMDC are known to be localized in the cytoplasm [1]. SAM is produced from methionine and ATP by S-adenosylmethionine synthetase (SÀÌS, EC 2.5.1.6.). It is of importance that SAM is not only the substrate for SAMDC providing aminopropyl for PA synthesis but also a basic donor of methyl groups for numerous reactions of transmethylation and a principal negative regulator of threonine and methionine biosynthesis.

Thus, four enzymes are involved directly or indirectly in the formation of Spd and Spm. SAMS is encoded by three, in dependence on plant species, isogenes *SAM1, SAM2,* and *SAM3* and they are expressed differently in response to ABA treatment, salt stress, and osmotic stress [40]. In the roots of tomato seedlings, these stressful factors increased the content of *SAM1* and *SAM2* mRNA, whereas only *SAM1* transcripts accumulated in the leaves. An increase in the level of *SAM* transcripts in tomato plants was not accompanied by a considerable accumulation of Spd and Spm. This means that mRNA formation was not a rate-limiting step of PA biosynthesis, and posttranscriptional, translational, or posttranslational regulation of gene expression was critical.

By basing on the data obtained for animals [41], it is commonly accepted that synthases of Spd and Spm in plants are also two different enzymes [1,42]. However, PAPT was isolated and partially purified only from maize and parsley [43,44], whereas SAPT was not still characterized at all [45].

Along with widely occurring (usual) PAs [41], prokaryotes are capable of the synthesis of unusual PAs. Thus, thermophilic and acidophilic bacteria living under extreme conditions contain aminopropyltransferase of wide substrate specificity. This permits them to synthesize PAs with a long polymethylene chain and increased number of NH_2 groups in the molecule (norspermidine, thermospermine, and caldopentamine) and also some branched PAs (hexamines and heptamines) [46-48]. Aminopropyl donor produced at SAM decarboxylation is used for unusual PA synthesis.

In some vibrions inhabiting sea water, norspermine was detected. The initial step of this PA formation is decarboxylation of 2,4-diaminobutyric acid; aminopropyl is used as a donor in the reaction of polymerization [49,50]. It is of interest that the same donor is used in the alternative homospermidine synthesis in cultured tobacco cells [32] and in *Lathyrus sativum* seedlings [51].

Plants are also capable of unusual PA synthesis. Norspermidine and norspermine were identified in *Canavalia gladiata, Vicia radiata* and *Vicia sativa* [52,53] and in cultured calluses of thermotolerant rice cultivar after heat shock (45°N, 30 min) [54]. Unusual PA formation in hyperthermia-tolerant calluses was positively correlated with ADC activity.

Unusual PAs (thermospermine, homocaldopentamine, and homocaldohexamine) were also found in osmotolerant alfalfa plants [55,56]. Their biosynthesis in this plant was catalyzed only by a single aminopropyltransferase (PAPT). This enzyme exhibited a high specificity for Put as an initial substrate but not for Spd or Spm as alternative initial substrates i.e., it functioned as a classical enzyme (PAPT) catalyzing Spd formation. This conclusion was confirmed by the inhibitory analysis. During the synthesis of usual (Spd and Spm) and unusual PAs in animals, aminopropyl was predominately used as a donor [41]. It is not clear whether this enzyme contains only a single protein or an enzyme complex with PAPT activity. It was supposed earlier that 1,3-diaminopropane (1,3-DAP) produced in the oxidative degradation of Spd and by polyamine oxidase might be a donor instead of propylamine during the synthesis of unusual PAs [55,56] because, in stress-induced plants, the biosynthesis of unusual PAs was, as a rule, accompanied by enhanced formation of 1,3-DAP [54, 56, 57]. The more so that in some bacteria and euglena, 1,3-DAP was a precursor of norspermidine [49]. However, as it was found by Bagga *et al.* [56]. PAPT recognized only Put as an initial substrate but not a structurally close diamine 1,3-DAP.

For the enzymology of PA synthesis, arabidopsis *acaulis5 (acl5)* mutant is of a great interest; the recessive mutation in the *ACL5* gene was manifested in the disturbance of stem elongation. This gene sequencing showed its high homology with sequences encoding the two enzymes of PA biosynthesis, PAPT and SAPT [58]. However, the synthesis of recombinant ACL5 protein in *E. coli* and its immunoenzyme analysis demonstrated that the *ACL5* gene encoded spermine synthase, although it might be that this protein displayed a wider substrate specificity, participating in the biosynthesis of other PAs as well [6].

It was shown for all living organisms that enzymes might function not only as separate moieties but also within multienzyme complexes where metabolites pass from one reactive center to another, so called 'substrate channeling' [59]. Such multienzyme complexes displaying a higher structural level than individual enzymes were called as metabolons. The formation of such complexes can improve stability and efficiency of enzyme partners, which is especially important for organism under extreme conditions [60,61].

Cadaverine

Cad is a relatively rare diamine in plants; it is derived from lysine, a byproduct of the aspartate pathway of methionine synthesis [62,63] (Figure 1). Cad synthesis from lysine is catalyzed by lysine decarboxylase (LDC, EC 4.1.1.18) via pyridoxal phosphate-dependent decarboxylation [64]. Under ornithine deficiency, ODC can use lysine as an alternative substrate for Cad synthesis. Such a possibility was demonstrated for yeast [65] and also a poplar hybrid cell line (*Populus nigra ' maximowiczii*) transformed with the mouse *ODC* cDNA [39].

In some legume species (*Lathyrus sativus*, for example), Cad may be produced by decarboxylation of either lysine or homoarginine via the intermediate production of homoagmatine [8] (Figure 1). It is supposed that both reactions are catalyzed by a single enzyme,

homoarginine-lysine decarboxylase, which differs from either LDC or ADC. An enhanced activity of this enzyme is evidently related to the fact that *Lathyrus sativus* is a producer of piperidine alkaloids (sparteine, lupinine, anabasine etc.) from Cad. In most plant species with a low Cad concentration in tissues, the activity of LDC is low. Under stress conditions, Cad accumulation in plants evidently compensates a decrease in the content of Put family of Pas [66-68].

As distinct from Put, Spd, and Spm, which are synthesized on thylakoid membranes [33], Cad is produced in the chloroplast stroma [69]. The genes for the enzymes of Cad biosynthesis are localized on the 5B chromosome of the wheat, whereas the genes for Put and Spm biosyntheses, on the 5A chromosome [16,17].

Some researchers reported that, in the halophyte *Pulicaria,* Cad was synthesized predominantly in roots [27], where, as it is known, plastids are represented by proplastids with the undeveloped membrane system. In another halophyte *Mesembryanthemum crystallinum,* the highest activity of LDC under salinity was detected in the root system as well.

The molecular mechanisms of Cad biosynthesis were studied for tobacco plants transformed with the gene encoding LDC from enterobacterium *Hafnia alvei*, which is capable of constitutive overproduction of the enzyme. Two different constructs were built for tobacco transformation [69]. In one of them, the gene was under the control of Tr promoter and did not contain a signal (transport) peptide. The second construct contained the promoter of the Rubisco small subunit gene and a signal sequence providing for the polypeptide transport into chloroplasts, a natural compartment of LDC. The results obtained showed that Cad synthesis in tobacco plants was enhanced only in the second case. When tobacco plants were transformed with the construct containing the gene for LSD from the enterobacterium *Hafnia alvei* under the control of the constitutive 35S CaMV promoter, root culture was obtained with a high LDC activity and increased level of alkaloids [70]. A direct correlation between the content of Cad and a capacity for alkaloid synthesis was also found for *Solanaceae* plants [8].

Molecular Mechanisms Controlling Polyamine Biosynthesis

Modern knowledge of the mechanisms of the PA synthesis regulation in prokaryotes and eukaryotes are considered in a number of comprehensive reviews [3,4,7,8,12].

The principal enzymes of PA biosynthesis are under the complex metabolic and developmental control and affected by stresses; such a control is necessary condition for the efficient regulation of cell metabolism. Thus, in transgenic rice plants overexpressing ADC, Put enhanced and Spd suppressed the activity of SAMDC, a key rate-limiting enzyme of Spd and Spm biosyntheses [71]. In one of rice cultivars, Spd and Spm accumulation was related to the enhanced expression of ADC [72], whereas in salt-tolerant rice and tomato plants, to enhanced expression of SAMDC [19]. It should be noted that the induction of SAMDC expression resulting in Spd and Spm accumulation is more often considered to be important for plant defense against stress than Put accumulation. SAMDC instability (half-life of 5-60 min) permits a rapid change in the amount of this enzyme in the cell and consequently, in the PA level in response to new conditions.

The enzymes of PA biosynthesis (ODC, ADC, and SAMDC) are controlled at transcriptional, translational, and posttranslational levels. Recently, Hu *et al.* [73] showed that

a 5'-untranslated leader sequence played a central role in both transcriptional and posttranscriptional control of SAMDC gene expression. This was related to the fact that in plants, as distinct from animals, this gene has no introns in the open reading frame but does have introns in the leader sequence, which are required for the enhanced SAMDC biosynthesis when the level of endogenous Spd is low.

It is believed that all enzymes of PA biosynthesis, including SAMDC are initially synthesized as inactive precursors (proenzymes), which are subjected to posttranslational processing with the formation of mature enzymes [74]. This process is very rapid and, as distinct from animal enzymes, is not controlled by Put. Thus, when ADC was synthesized in oat plants, a 66-kD precursor was first synthesized; it was subjected to a limited proteolysis with the formation of two polypeptides (44 and 22 kD); thereafter, active enzyme form was produced by the binding of these polypeptides via a disulfide bridge [75].

It is of interest that, in detached oat leaves subjected to osmotic stress, processing of ADC inactive precursor was inhibited by Spm treatment [33,76]. The authors believe that, when Spm is absent, transcription of ADC gene is enhanced, and inactive ADC precursor is synthesized, which is processed into the active ADC form. ADC catalyzes Put synthesis. In the presence of exogenous Spm, the level of mRNA increased but the number of active enzyme molecules decreased and Put accumulation was blocked. Nam *et al.* [77] reported that, in legume plants (*Glycine max*), ADC apoprotein consisted of three identical subunits. Evidently, the type of posttranslational modification of the proenzyme could somewhat differ from that described for oat plants [33].

In some plants, *Arabidopsis* for example, Put can be synthesized only by ADC. The gene encoding ODC and corresponding proteins were not found in this plant [2]. However, *Arabidopsis* like some Brassicaceae plants contains two genes for ADC (*ADC1* and *ADC2*) [78]. By the methods of molecular biology, it was shown that mechanical injury to arabidopsis leaves or their treatment with jasmonic acid resulted in the activation of only a single gene for Put synthesis, the *ADC2* gene [79]. This was accompanied by a transient increase in the content of Put but not Spd or Spm. It is of interest that the sequences of ADC1 and ADC2 proteins were identical by 80% and differed only in their terminal fragments. It might be that these proteins differ in their localization, as it was shown for ADC in oat plants [33], and fulfill different biological functions [79].

Stress-induced signals play an important role in the control of PA synthesis by abiotic and biotic factors; these signals activate or inhibit the operation of signal cascades and transcription factors. The earliest plant response to stress is believed to be a generation of reactive oxygen species (superoxide anion and hydrogen peroxide), which mediate in signal transduction. Moreover, PAs themselves and the products of their oxidative degradation (H_2O_2) can induce expression of the genes for oxidative enzymes, peroxidases and superoxide dismutases [80,81].

Polyamine Catabolism

PA catabolism is an efficient regulator of the free PA level in the cell; its products can fulfill an important physiological role under both normal and stress conditions [12,82-84].

Polyamine degradation in plants is catalyzed by the two oxidative enzymes: copper-containing diamine oxidase (DAO, EC 1.4.3.6) and flavoprotein-dependent polyamine oxidase

(PAO, EC 1.5.3.3) [85]. It was believed that the highest DAO activity was characteristic of legumes, and the highest PAO activity for grasses. Now, this opinion is reassessed because DAO activity was found in grasses as well [86]. Both enzymes are localized in the cytoplasm and cell walls where they provide hydrogen peroxide required for suberinization and lignification, which confer firmness to the cell walls [87,88].

DAO catalyzes oxidation of primary amino groups in many biogenic amines, including mono-, di-, and polyamines with Put and Cad as most preferable substrates. As a result of DAO-catalyzed oxidation of Put, Spd, and Spm, amino aldehydes, hydrogen peroxide, and ammonia are produced. PAO catalyzes oxidation of secondary amino groups of Spd and Spm (but not other PAs) with the formation of H_2O_2, 1,3-DAP, and amino aldehydes, 4-aminobutyraldehyde and 4-(3-aminopropyl)-aminobutyraldehyde. The same amino aldehydes are produced as a result of Put and Spd oxidation by DAO. A terminal product of this oxidation, 1,3-DAO, can be a substrate for DAO-catalyzed β-alanine formation.

First reports about enzymes of PA catabolism and corresponding genes appeared in the early 1990s. DAO was isolated and purified to the homogenous state from barley plants [89]; later, the changes of its activity under stress conditions were studied [86]. In pea plants, DAO comprises 0.1% of soluble protein [90]. This enzyme is a homodimer. Each DAO subunit contains one copper atom and a quinone cofactor. cDNAs of pea DAO were obtained, and, using Southern hybridization, the genes for DAO were identified in many mono- and dicotyledonous plants [91]. By Northern-blot analysis, it was demonstrated that the level of DAO mRNA increased in darkness, which was correlated with a high enzyme activity. On the other hand, anoxia, low temperature, and other stressors did not affect DAO activity, and the oxygen concentration did not control a transcriptional activity of the DAO-encoding gene [92].

Recently, some reports appeared demonstrating that N^1-acetylation preceded oxidative PA degradation in plants [8,37,93]. In animal cells, increased Spd and Spm syntheses are often accompanied by increased catabolic breakdown of these compounds via the induction of Spm acetyltransferase and PAO activities [94].

Acetyl polyamines were identified in sugar beet seedlings [95], in chloroplasts of Jerusalem artichoke leaves (Del Duca *et al.* 1995), in maize roots [37], and various *A. thaliana* organs [8]. This pathway of PA degradation involves various enzymes, including PAO. In plants, both anabolic and catabolic enzymes can play a role in the regulation of PA levels during the cell cycle and cell division/expansion processes [11,84].

Spontaneous cyclization of amino aldehydes derived from Put results in the formation of 1-pyrroline, which is converted into γ-aminobutyric acid (GABA) by pyrroline dehydrogenase; GABA is a potential modulator of many physiological processes [96]. However, most of GABA accumulated due to glutamate oxidation in stressed plants. GABA formation in the process of Put oxidative degradation was studied in detached leaves of *Glycine max* [97]. Using the inhibitory analysis, these authors established that GABA (20 mM) introduced into the leaves induced PA (Put, Spd, and Spm) accumulation via the activation of the ADC pathway. It is well known that, in plants, GABA is metabolized by GABA transaminase and succinyl semialdehyde dehydrogenase into succinate, which enters into the Krebs cycle [98]. By basing on these data, we can suppose that GABA carbon, being involved in the Krebs cycle, enhances the formation of glutamate, which, in its turn, accelerates PA biosynthesis.

Pulse-chase experiments with 1,4-^{14}C-Put introduced into the roots of a halophyte *Limonium tataricum* demonstrated rapid metabolization of this diamine into GABA via the DAO pathway [57]. Moreover, the incorporation of ^{14}C into GABA was detected after the introduction of labeled Spd into the roots, which indicated the possibility of Put formation via PAO-catalyzed degradation of Spd and a further conversion of diamine into GABA. The activity of enzymes involved in Put catabolism, the pathways of its oxidative degradation with the formation of secondary metabolism products, and their effects on the intracellular PA pool depend on plant species, their developmental stage, and environmental conditions.

As distinct from Put, amino aldehyde, an initial product of oxidation of Cad terminal amino groups, is further converted into 1-piperedine, a precursor of alkaloids. Amino aldehydes are the products of oxidation of the two terminal amino groups in Spd; they are converted into either the equilibrium mixture of two cyclic compounds, 1-(3-aminopropyl)pyrrolinium and 1,5-diazabicyclononane, or into putreanine and isoputreanine [57].

Investigations of PA catabolism have been focused mainly on changes in their levels and spectra, leaving the biological significance to be determined. Paschalidis and Roubelakis-Angelakis [84] presents the sites and regulation of PA catabolism referred to cell division/ expansion, cell cycle progression, and vascular development in tobacco plants. Gene expression and immunohistochemical analysis revealed that, DAO and PAO in developing tissues precede and overlap with nascent nuclear DNA and also with peroxidases and lignification. The specific activities of the enzymes of PA catabolism increased basipetally in the leaf central and basal, petiolar, and internodal regions throughout development. PAO activities and protein levels increased with ontogenic stages. Moreover, *pao* mRNA levels dramatically increase with age (more than 10-fold from the youngest to the oldest leaves), suggesting that changes in *pao* expression are mainly regulated at the transcriptional level.

The results obtained by Paschalidis and Roubelakis-Angelakis [84] permit a supposition that, in stressed plants, developmental changes in PA catabolism are enhanced because DAO and PAO expression and H_2O_2 production occur in the cells destined to undergo lignification.

Polyamine Transport

In order to understand the physiological role of PAs or any other biologically active compounds, their capacity of interorgan transport should be considered. Tens of years, researchers stated with certainty that a PA polycation nature is incompatible with their long-distance transport. The more so, that, in one of the first studies using ^{14}C-PAs, it was shown that Put and Spd were very poorly transported within the plant. Therefore, it was concluded that PAs could not be growth-regulating compounds and could not exert distant action, as distinct from phytohormones; they were believed to be local modulators of metabolism in the regions of their increased biosynthesis [99]. It was admitted that, in experiments with labeled PAs, their limited spreading along the plant might be an artifact related to the problems of isotop dilution, metabolization, conjugate formation, and PA possible binding to the cell components, including the components of the transport systems.

However, it was noted later that PAs absorbed by plant tissues could be transported over long distances [100]. The presence of large amounts of PAs in the xylem sap and phloem exudates, which was detected firstly by Friedman *et al.* [101] and then in our exper nents

[67,68], is a good argument for interorgan PA transport. Put, Spd, Spm, and Cad were identified in the phloem sap and extracts from rice stems [102]. The authors concluded that diamines, Put and Cad, were easily transported within the phloem, whereas Spd and Spm transport was rather limited. It was supposed that the capability of PA transport along the phloem decreased with increased number of amino groups in the molecule.

It is of interest that stress factors, such as potassium deficiency, acidic pH, and salinity, enhanced a PA interorgan transport [101-104]. When *M. crystallinum* L. organs were subjected to local 2h heat shock, we observed Cad and Put translocation in acropetal direction along the xylem and in basipetal direction along the phloem [67,68]. Among rapidly transported PAs, Cad played an especial role; as distinct from Put and Spd, it accumulated in leaves of the common ice plants in response to salinity and ethylene treatment. The interrelation between ethylene-dependent Cad formation with its subsequent stress-induced transport to roots and the functioning of the system of ethylene signal recognition was shown in experiments with the *A. thaliana ein4* mutant insensitive to exogenous ethylene. On the ground of experiments performed, a hypothesis was put forward that stress-induced induction and interorgan translocation of Cad was under ethylene control, which formation was characteristic of the plant response to short-term hypothermia or salinity [67,68]. This means that stress phytohormones, such as ethylene and maybe ABA, could trigger the interorgan PA translocation in plants.

The mechanisms of transmembrane PA transfer in plants are only poorly studied. First investigations of this problem were performed with *E. coli* cells [105]. It was established that PA uptake by *E. coli* cells demanded energy, and two transport systems were involved in this process: one system for Put and another for Spd and Spm.

E. coli mutants deficient in the PA transport and clones harboring the genes for PA transporters were used for investigation of the molecular properties of the PA transport systems. It was established that, in these mutants, a periplasmic transport system was involved in PA uptake by bacteria; this system was controlled by the two genes (*pPT104* and *pPT79*) for Put and by a single gene (*pPT104*) for Spd. This transport system comprised four protein types (potA, potB, potC, and potD) differing in their localization in the periplasmic space. Expression of all four genes and the synthesis of all four proteins were required for the highest transport activity of this system. The molecular analysis of the *E. coli* system for PA transport permitted a creation of a model of the secondary structure of two transporters and identification the site of Put interaction with a transporter; the mechanisms of regulation of PA synthesis, uptake, and excretion in bacteria were supposed for the first time [106].

Recently, several reports appeared simultaneously about identification and the mechanisms of functioning of protein transporters in eukaryotic cells (*Saccharomyces cerevisiae)* [107,108]. The team of Japanese researchers [107] established that Gap 1p transporter was localized in the plasma membrane and catalyzed Put and Spm uptake. The two other proteins, TPO1 and TPO5, catalyzed PA excretion [107,108], which was performed by TPO1 at acidic pH (5.0) [107]. TPO1 transport activity increased after its phosphorylation by Ser19 protein kinase C and Thr52 casein kinase. TPO5 transporter encoded by the *YKL174c* gene was resistant to high PA concentrations (120 mM Put and 3 mM Spd). It was more efficient in the transport of Put than Spd. In *S. cerevisiae,* Aouida *et al.* [109] identified a permease with a high affinity for Put and Spd, which was identical to Agp2p permease catalyzing transport of a set of amino

acids. Deletion of the *AGP2* gene reduced sharply the initial rate of Put and Spd uptake and conferred a high resistance toward exogenous PAs to a transporter. *AGP2* is the first gene encoding eukaryotic permease with a high affinity for Spd, which plays a key role in PA uptake by yeast cells.

It was also reported that, in yeast cells, the *YLL028* gene was identified encoding a vacuolar transporter specific for PAs [110]. The cells transformed with this gene acquired a resistance to PA toxicity, which was suppressed by Bafilomycin A1, the inhibitor of vacuolar H^+-ATPase. In these cells, the vacuolar membrane displayed a highest capacity for PA transport. Some evidence was presented indicating that the membrane protein encoded by the *YLL028* gene was a PA transporter of the tonoplast.

As distinct from prokaryotes and yeast, molecular investigations of plant PA transport systems are early in their development. First studies [110,111] were destined to the kinetics of Put transport. Published data are very difficult to interpret because of an extremely high concentration of exogenous diamine (up to 100 mM) and usage of some plant systems (cell suspension, protoplasts, or detached flower petals) that could have transport systems distinct from those in intact plants and tissues. Di Tomaso *et al.* [112] presented more complete and correct data on the kinetics of Put transport, its subcellular distribution, and excretion, which were obtained on intact maize seedling roots. According to the results of these authors, roots absorbed 0.05 and 1.0 mM Put linearly for 30 to 40 min; the rate of its uptake was 0.35 μM/g fr. wt. Initially, Put penetrated into the root apoplast, followed by transport across the plasma membrane. These reports suggest that a portion of the exogenously applied Put is metabolized in maize root cell walls by DAO, but the bulk of the Put is transported across the plasmalemma by a carrier-mediated process, similar to that process for animal systems. It was also shown that Put accumulated in the root-cell vacuoles, which served for this PA storage. From the vacuole, Put could be transported back across the tonoplast and plasma membrane into the apoplast of the cortex and epidermis cells.

A series of studies performed by Italian researchers [113,114] was destined to general principles of PA specific binding to plasma membrane proteins. This binding can fulfill a dual role: early event of PA signal recognition or binding to a specific transporter. In order to choose between these two possibilities, plasma membrane vesicles were isolated from etiolated pumpkin hypocotyls and two Spd-binding proteins (44 and 66 kD) were extracted from them and purified by gel filtration through G-200 Sephadex. No activity of the enzymes of PA biosynthesis (ADC and ODC) was found in vesicles; in contrast, a considerable activity of vanadate-sensitive ATPase, a marker of the plasma membrane, was detected, but this activity was not eluted together with Spd-binding proteins during gel filtration. The authors do not exclude a possibility that such an ATPase activity could correlate with specific Spd binding at the plasma membrane.

Specific Spd binding to membrane proteins was detected in etiolated maize (*Zea mays*) coleoptiles [115]. At the final step of Spd-binding protein purification by liquid chromatography and gel electrophoresis, it was shown that it comprised a large polypeptide of 60 kD and a specific Spd-binding polypeptide of 18 kD. Using various methods of molecular biology, it was shown that mRNA for 60- and 18-kD polypeptides was evidently produced by alternative splicing. It was elucidated that Spd-binding sites of protein must contain at least three negatively charged groups with distances between them corresponding to the distances between positively charged groups in the Spd molecule. Such an arrangement of the Spd-

binding site of the protein is likely universal because it was found earlier in the PA-binding PotD protein of *E. coli* [116].

Polyamine Physiological Role

Below, we consider some approaches used for the investigation of PA physiological role.

Usage of the Inhibitors of PA Biosynthesis

Until recently, a principal tool for deciphering regulatory mechanisms of PA metabolism was the usage of chemical inhibitors; most of them were initially used in human cancer chemotherapy to suppress PA accumulation in tumors. Most widely used inhibitors of various enzymes of PA biosynthesis and catabolism are difluoromethylornithine (DFMO) for ODC, difluoromethylarginine (DFMA) for ADC, methylglyoxalbisguanylhydrazine (MGBG) for SAMDC, cyclohexamine (CHA) for Spd synthase, aminoguanidine (AG) for DAO and others. The usage of inhibitors in PA metabolism studies permitted a considerable advance in the understanding of plant stress-tolerance due to the possibility to switch off separate stages of their biosynthesis [4,7,94].

The application of DFMA and DFMO help to establish that ADC pathway operates in the constitutive Put synthesis under normal conditions, whereas under stress conditions (osmotic and salt stresses), both ADC and ODC pathways could be activated, resulting in Put accumulation. However, the accumulation of major PAs, Spd and Spm, depended only on ODC activity [7,66,117]. These studies showed that PAs were involved in the wide range of physiological processes: development, cell division and expansion, somatic embryogenesis [12,26,118]. The usage of inhibitors permitted the elucidation of compensatory reaction accompanying the switching off some PA biosyntheses, which is of importance for understanding the mechanisms of plant-cell homeostasis, especially under stress conditions [119]. Nevertheless, some limitations of the inhibitory analysis should be mentioned: their possible metabolization in tissues, differences in the rates of their uptake, insufficient specificity determined frequently by differences in the localization of the inhibitor and a target enzyme, injurious effect on membranes and other drawbacks [6,39].

Mutants Displaying Changed Polyamine Metabolism

One of genetic approaches for the investigation of the mechanisms of PA signal perception and transduction in stressed plants is biochemical and physiological analysis of mutants displaying different phenotypes.

At present, several types of plant mutants with induced changes in PA metabolism were obtained. Among them, mutants of tobacco, petunia, tomato and arabidopsis deficient in PAs and the genes of their biosynthesis and mutants resistant to PAs and the inhibitors of their biosynthesis. Kakkar and Sawhney [3] review the list of mutants and characteristics of their phenotypic and biochemical defects.

Mutant tobacco lines resistant to MGBG are of interest for establishing PA morphogenic role. These mutants display dwarfism and changed morphology of floral organs; they manifest enhanced SAMDC activity and have the expanded PA pool [120]. In petunia, mutant line with changed flower morphology also showed a high level of endogenous PAs and enhanced ADC

activity [121]. In the leaves of non-flowering tobacco *rmb7* mutant, PA conjugates were not found, which are supposed to be transported to stem apices toward floral buds and induce flowering [122].

Some types of mutants are beneficial for studying the PA role in stress physiology. Thus, tobacco DFMO-resistant mutant with a high PA concentration was resistant to low pH values inducing an acidic stress in plants [123,124]. The *flacca*-ABA-deficient tomato mutant is characterized by a high ADC and low ODC activities at late developmental stages, which was accompanied by the reduced total level of PAs. Such a mutant is of importance in the study of interactions between ABA and PAs during adaptation to abiotic factors [125].

Recently, the group of Japanese researchers described arabidopsis insertion mutants harboring T-DNA for two genes of Spd synthase, *SPDS1* and *SPDS2* [126]. While each mutant allele showed normal phenotype, *spds1-1 spds2-1* double-mutant seeds were shrunken and have embryos that were arrested morphologically at the heart-torpedo transition stage. This mutation was lethal. These seeds contain a reduced level of Spd and, in contrast, a high level of Put. These data provide the first genetic evidence indicating a critical role of the Spd synthase in plant embryo development. On the basis of these data, we may suppose that a double coding of PA synthesis enzymes in higher plants is essential for plant survival under extreme conditions. At the same time, Imai et al. [126,127] showed that, as distinct from Spd, Spm was not necessary for arabidopsis normal development. Earlier, it was shown that a disruption of the *ACL5* gene, encoding Spm synthase in arabidopsis and required for stem elongation resulted in a severely dwarfed phenotype [58]. However, exogenous Spm could not restore normal stem growth. The authors believe that this is explained by the fact that exogenous Spm did not reach a required intracellular compartment or did not produce a conjugate required for the manifesting of its action.

Transgenic Plants as a Model for Studying Polyamine Biological Role

At present, other approaches became available for studying the mechanisms of PA biosynthesis as well. One of the promising approaches is the production of transgenic plants harboring the genes encoding enzymes of various pathways for PA biosynthesis. In Kakkar and Sawhney [3] review, the list of genes controlling PA metabolism in plants, which were characterized and cloned, is presented.

Since the 1990s, studying transgenic plants help to answer some important questions concerning the control of PA metabolism. Firstly, overexpression or negative regulation of key genes for GDC, ADC, and SAMDC permitted a control of a Put endogenous level. Overexpression of yeast ODC cDNA in tobacco plants [128] or mouse ODC cDNA in tobacco and carrot plants [129,130] increased the level of Put but did not affect the levels of Spd and Spm, as compared to wild-type plants. At the same time, transgenic tobacco leaves expressing human SAMDC cDNA contained much more Spd and Spm and reduced amounts of Put. SAMDC overexpression in transgenic rice plants was accompanied by Spd accumulation and improved salt-tolerance as compared to wild-type plants [71]. Transgenic rice plants expressing *Datura stramonium* ADC under the control of the monocot *Ubi-1* promotor produced a much higher level of Put under drought stress, only promoting Spd and Spm synthesis and ultimately protecting the plants from drought [131].

When antisense SAMDC cDNA was inserted into the potato genome, Spd production was sharply reduced, and transgenic tubers displayed a changed phenotype [9,132,133]. In transgenic tobacco plants transformed with the ADC gene from oat under the control of an inducible promoter (Tet-repressor system), an increased levels of this gene transcript, ADC activity, and free Put were observed [135]. Transgenic plants displayed a changed phenotype: necrotic lesions appeared on their leaves, and growth was retarded, which was induced by a high level of endogenous Put toxic for plant growth and development. On the other hand, antisense potato transgenes harboring SAMDC cDNA under the control of 35S promoter of cauliflower mosaic virus displayed an abnormal phenotype (growth retardation, non-flowering plants, leaf chlorosis, etc.) on the background of a decreased SAMDC transcript level, reduced enzyme activity and Put level but an enhanced ethylene evolution [132]. All attempts to obtain transgenic plants with SAMDC construct in the normal orientation were unsuccessful. This permitted a supposition that constitutive overexpression of this enzyme might be lethal [132]. At the same time, in order to elucidate specificity in the metabolism and development regulation by PAs, it is necessary to change the PA level in various tissues just by expression of sense and antisense constructs under the control of tissue-specific promoters [12]. In general, the levels of Spd and Spm in cells are least changeable because of the functioning of homeostatic regulation [6], which might be related to the supramolecular organization of enzymes involved in their biosyntheses [39].

Functioning in plants of two alternative pathways of Put biosynthesis does not exclude a dependence of their regulation on mutual intracellular conversions of their substrates (ornithine and arginine) or their availability. Experiments with transgenic cell line of *Populus nigra* × *maximowiczii* plants transformed with mouse *ODC* cDNA was destined to elucidate these questions [39]. In this study, a capability of plant cells overexpressing a foreign *ODC* gene to maintain a high level of Put via switching on the homeostatic mechanism was demonstrated. This mechanism induced an increased production of ornithine and its precursor glutamate at increased activity of ODC. Earlier, it was shown that transgenic animals, which could not tolerate excessive production of Spd and Spm in their cells, excreted their precursor Put, i.e., PA overproduction induced the cell homeostatic response [134,136]. In addition, using a transgenic system, it was demonstrated that plant ODC could use as a substrate ornithine synthesized directly from glutamate rather than ornithine produced from arginine in the urea cycle. Thus, the usage of transgenic plants helps to decipher compensatory mechanisms in the PA metabolism, which could play a great role in the maintenance of PA homeostasis required under stress conditions. The discussed above ethylene-induced accumulation of Cad in stress-tolerant common ice plants can be interpreted in a similar way.

In the opinion of some workers, some inconsistencies arising during PA studies with the usage of transgenic plants can depend on various factors: transgene source, effect of position, plant material for transformation, promoter type, and others [6]. PA accumulation in tissues differing in metabolic activity was studied [137]. In general, more PAs was accumulated in tissues of a lower metabolic activity. More significant results concerning the control of transgene expression were obtained with inducible or tissue-specific promoters [138].

Polyamines and the Control of Cell Cycle

PAs, being the components of eukaryotic and prokaryotic organisms, are important for the

control of cell divisions and cell differentiation, which is determined by their role in such cell processes as replication, transcription, and translation. Such unique PA functions are related to their structural organization, *i.e.*, regular spatial distribution of positive charges in the molecule, which distinguish them from Mg^{+2} and Ca^{+2} with point charges.

The inhibition of PA biosynthesis is known to result in the cell cycle arrest at the G_1 phase, suggesting the involvement of PAs in DNA synthesis, which is accompanied by retardation or stopping cell growth [139].

An increased PA content during the presynthetic period and synthetic period (S) preceding the premitotic period (G_2) and mitosis (M) is a universal phenomenon in all eukaryotes [7]. An increased level of endogenous Spd was found during the G_1 phase of the cell cycle in the cells on potato tuber sections preliminarily treated with 2,4-D to release dormancy [140,142]. Treatment with exogenous Spm enhanced mitosis commencement in the embryo axes of pea seeds [142], whereas the inhibition of Spd and Spm biosynthesis arrested cells in the G_1 phase [143]. Disturbed embryo development was observed in arabidopsis plants with mutation in the *Spds* gene [126]. A close interaction between PAs and cell division was found also for potato tubers treated with the inhibitors of PA synthesis [144,145]. A requirement of PAs for cell proliferation can be ascribed to their capability of specific binding to DNA and chromatin for the maintenance of their conformation and transcriptional activity. A high percentage of PA binding to nucleic acids during G_1 phase argues for this hypothesis [141]. In the culture of protoplasts from cereal leaves, which were not capable of the synthesis of DNA in sufficient for their division amounts and therefore, were arrested in the G_1 phase of the cell cycle, the addition of Cad, Spd, and Spm to the nutrient medium induced DNA synthesis and eliminated the block of division. The inhibitors of Spd and Spm biosynthesis, similarly as osmotic shock, blocked a transition of pea root meristematic cells from G_1 to S phase. However, the addition of exogenous PAs restored the normal course of the cell cycle [7,146]. Earlier, in *Pisum sativum* plants subjected to NaCl salinity, the accumulation of free Cad was observed [147]. In meristematic cells of pea root tips subjected to salinity, considerable ultrastructural changes were observed in nuclei during 10 days: primarily in the number and volume of nucleoli and several fold increase in the content of DNA per nucleus, that is, some signs of polyploidization were evident [147]. An increase in the number and volume of nucleoli under salinity was evidently related to a short-term stimulation of rRNA by PAs. A considerable stimulation of protein synthesis in wheat root cells under chlorine salinity was noted in other studies [148]. When pea plants were transferred to salt-free medium, the normal structure of nuclei was restored. Thus, observed changes in the number and size of nucleoli were reversible, which evidently could be considered an adaptive nucleus response to stressor action [147].

Similar changes were found in suspension cells of salt-resistant tobacco line characterized by a transient accumulation of Put and Spd under 170 mM NaCl [21]. The content of DNA in the nuclei of these cells changed as compared to salt-sensitive line. Thus, in salt-resistant cells, the nucleus was almost twice smaller, but the content of DNA in it remained essentially unchanged. In salt-sensitive cells in the presence of NaCl, the nucleus contained mainly haploid DNA, whereas the nucleus of salt-resistant cells contained diploid and polyploidy DNA, that is, most cells of salt-resistant line were in G_1 and S phases of the mitotic cycle and were ready for proliferation. The data obtained indicate that PA accumulation in salt-resistant cells could initiate adaptive rearrangements in nuclei favoring mitosis commencement.

Almost three decades ago, the absolute requirement of PAs for proliferation of animal cells was well characterized by their capability of affecting DNA and chromatin conformation [151]. In the *in vitro* cell systems, PAs changed both DNA conformation (from B to Z) and the structure of chromatin and nucleosomes [151,152]. Such specific PA-induced changes in cells are not consequences of the maintenance of ionic homeostasis in their presence, which was proven by the addition of bivalent cations (Mg^{2+}) into medium. Treatment of isolated nuclei with Spd abolished a block of cell proliferation induced by application of specific inhibitor of ODC biosynthesis (DFMO) [152]. Moreover, in experiments with isolated nuclei of the cell line of human cancer cells, PAs were shown to control transcription of some genes specific for tumor development [150]. In addition, experiments with isolated nuclei showed that PAs could affect numerous molecular mechanisms not only at the level of transcription, but also at posttranscriptional and posttranslational levels.

All considered molecular aspects of PA interaction with nuclear DNA and RNA changing their efficiency depend primarily on PA capability of producing ionic and less frequently covalent bonds with nuclear macromolecules. Changes in the intranuclear pool of PAs have often a decisive role in the modulation of nuclear gene expression [153]. PA deficiency due to their leakage during nucleus isolation or after long-term action of the inhibitors of their synthesis induced a nucleus response, namely, enhancement of mRNA synthesis for ODC, a key enzyme of PA synthesis in animal cells. Not only ODC gene expression but also the mechanism of its amplification was switched on [154]. Some researchers reported that nuclear PAs comprised mainly Spd and Spm; the geometry of NH_2 groups in these PAs matches best to negatively charged groups of DNA, RNA, chromatin, and proteins. Therefore, PAs in the nucleus fulfill an important function, maintaining the conformation of informational centers and their protection against endonucleases. PAs suppress digestion of internucleosomal linker DNA by endonucleases, induced by some anti-cancer preparations, and this opens an additional field for PA possible action as physiological blockers of apoptosis [94].

However, the role of PAs in such specific biochemical processes important for cell growth requires the investigation of molecular mechanisms of PA action on transcription of the genes of early proliferative response. Most important studies in this area were performed for human tumor cells, which differ from healthy cells by a high content of PAs and high activities of enzymes of their biosynthesis, ODC primarily. This is often accompanied by enhanced transcription of specific genes associated with cell growth [152,155]. An increased level of endogenous PAs in tumor tissues was detected very early, in the first studies of PAs in living cells, firstly in animals and later in plants [156]. Thus, tumor tissue of the roots of *Scorzonera hispanica* plants differed from normal tissue by a high content of Put and Spd and was characterized by almost 100-fold increased activities of ODC and ADC.

Cell entering mitosis is known to depend on the activity of multienzyme complex comprising cyclin and protein kinase [157,158]. Protein kinase activity in wheat leaves decreased under water deficit [159]. During transition of sugar beet suspension cells from dormancy to proliferation, Spd and Spm, 6 h after their addition, could induce expression of *Bvcyc* II gene encoding one of mitotic cyclin subunits [160]. This effect indicates that PAs could be considered as effective mitogenic stimulators.

There are some reports that Spd and Spm could be involved in the mechanism of cytokinesis of animal cells, facilitating the formation of the actin-containing contractile ring. In Chinese

hamster cell culture, the formation of actin microfilaments and microtubules occurred only in the presence of PAs, and the inhibition of Spd and Spm biosynthesis blocked cytokinesis [161,162]. These data demonstrated a functional interaction between PAs and cytoskeletal structures in the cell cycle.

Polyamines as Second Messengers

PAs are supposed to mediate phytohormone signaling, *i.e.*, fulfill the role of second messengers [3,26,163,164]. Experimental evidence for this PA function was first obtained for animal cells. As early as in the 1983, it was shown for the first time for mouse kidney cortex that a transient PA (Put, Spd, and Spm) accumulation induced by an animal hormone testosterone generated a Ca^{2+} signal via its enhanced exit into the cytoplasm from the reserve membrane pool [165]. Later, in the work with cultured animal cells, it was shown that, along with the control of intracellular Ca^{2+} level, PAs were involved in the hormonal signal transduction via their binding to G-proteins, which activated hormone recognition by the receptor [166]. Spd and Spm could function as blockers of potassium channels in the plasma membrane and ionic channels in the tonoplast [167].

The role of PAs as second messengers in plants was recently demonstrated in the series of studies by Messiaen *et al.* [168-171]. These authors were based on the presence of PAs in the cell walls, where they produced complexes with acidic polysaccharides (pectins); these complexes were considered earlier as one of the factors in the control of pH, thus affecting cell expansion [172], or in the control of methylesterase activity in the cell walls [173]. It was also known from some studies that pectin fragments (α-1,4-oligogalacturonides), which formation is catalyzed by methylesterases, were capable of modulation of various morphological and physiological processes in the cell walls and at the level of the whole plant, in particular in defensive responses [174,175]. In the laboratory of Messiaen [173], it was demonstrated in cultured carrot cells that a low concentration (10^{-6} M) of a pectin fragment produced a calcium-induced favorable supramolecular conformation, which was recognized by cells as a signal molecule controlling lignification and hydrogen peroxide generation in the cell wall matrix. Messiaen and Van Catsem [169] supposed that pectin-PA complexes produced in the cell walls helped recognition of pectins by methylesterases. However, in experiments on PA binding to isolated carrot cell walls and to polygalacturonides, it was found that PAs (Spd^{3+} and Spm^{4+}) with a high affinity for galacturonides and Ca^{2+} blocked the formation of Ca^{2+}-induced supramolecular conformation of pectin fragments, underlying their signaling activity. The results obtained indicate that plant PAs could function as second messengers modulating pectin signal transduction and thus affecting various morphological and physiological processes in the cell walls and protoplasts of plant cells.

It was recently shown that, in tobacco leaves, Spm could be a messenger in the activation of protein kinases by salicylic acid or wounding [176,177]. Spm-induced activation of MAP kinases and wounding-induced protein kinase was abolished by leaf pretreatment with antioxidants and blockers of Ca^{2+} channels in mitochondria.

However, PA involvement in the maintenance of plant growth and development and their interaction with phytohormones was not studied properly at the molecular level. Recently, Hanzawa *et al.* [58] isolated the *ACL5* gene required for internodal elongation of the arabidopsis

stem and gibberellin signaling pathway; simultaneously, this gene encoded proteins with PA-synthesizing activity. Thus, in the model system studied, PAs could support phytohormone action as a component of their signaling pathways, and therefore, they are considered second messengers in accordance with previously made statements [3,99].

In recent years, some reports appeared about more complex character of interaction between some phytohormones and PAs [178]. Thus, it was shown that PAs could block rapid cytokinin-induced effects based on expression of the genes of cytokinin primary response [178]. In this work, amaranth seedlings accumulating betacyanine in response to cytokinin treatment and transgenic arabidopsis plants harboring the reporter *GUS* gene under the control of cytokinin-dependent P_{ARRS} promoter were used as model systems. In both systems, all PAs tested (Put, Spd, Spm, and Cad), especially Put and Spm, inhibited the accumulation of amaranthine and activity of the *GUS* gene induced by 5 μM benzyladenine. The PA action manifested at the posttranscriptional level, not affecting the cytokinin-dependent mRNA accumulation. These data showed that PAs did not behave as second messengers of cytokinins in the model system used by the authors, as distinct from earlier suppositions, and did not affect total membrane receptor protein, as was supposed in the work of Naik *et al.* [163]. In the authors' opinion, the physiological role of PA-induced inhibition of cytokinin effects could be in the compensatory regulation of the intracellular cytokinin content when their concentration became excessive. This mechanism can operate under conditions of plant adaptation to extreme conditions when the retardation of growth processes is required for plant survival [179].

Polyamines as the Regulator of Plant Growth and Development

Polyamines are one of the classes of low molecular weight compounds capable of modulation of many important processes of plant growth and development at various stages of their ontogeny under both normal and stress conditions. They are involved in the initiation of cell division and expansion in plant morphogenesis, flowering, and senescence [26]. Simultaneously, all these processes are under hormonal control as well. Some evidence indicates the interaction between PAs and phytohormone regulatory systems [180-183]. Most PA physiological functions resemble those of cytokinins [14].

In the first years after PA detection in plant cells and the onset of active investigations concerning their physiological role, a great attention was paid to their capability of maintaining a high growth activity at definite developmental stages. Some early reports presented data about growth effects of endogenous and exogenous PAs at the level of intact plants, which resembled the effects of phytohormones [164]. In these studies, a great attention was paid to direct and indirect interaction between PAs and growth-inducing phytohormones. Thus, in some plants, auxin, gibberellin, and cytokinin stimulated biosynthesis and increased the content of PAs, whereas exogenous PAs affected the level of endogenous phytohormones [180]. One of the first data concerning auxin effect on the content of Spd and Spm in plants was obtained for *H. tuberosus* tubers after IAA-induced release of their dormancy [184]. In one of the recent works performed with arabidopsis, it was shown that IAA induced the *ACL5* gene encoding Spm synthase, but ABA or gibberellic acid could not induce this gene [58]. Inactivation of this gene retarded stem elongation and suppressed cell expansion.

In spite of the fact that PAs are relatively homogenous group in their chemical nature, i.e., they are organic hydrophilic cations differing only in the number and position of amino groups in their molecules, they can be divided into two groups based on their biological effects. Put and Cad stimulate cell expansion and root formation like auxins and gibberellins [4,26,180,185,186], whereas Spd and Spm regulate cell division, organogenesis, and senescence like cytokinins [26].

The level of endogenous PAs in the plant cell is much higher than that of phytohormones: their content varies from nanomoles to millimoles. High PA concentrations are usually present in actively growing plant tissues and during somatic embryogenesis. It was shown for cultured carrot cells, which were transformed with mouse ODC cDNA and had an increased intracellular Put concentration, that the induction of embryogenesis became possible at the deficiency of auxin [130]. Thus, Put was capable of manifesting a typical auxin effect. Changes in PA metabolism during somatic embryogenesis were studied in various plant systems [187-189]. In the recent study of Bertoldi *et al.* [28], the content of PAs, activities of the enzymes of their biosynthesis and the transcriptional regulation of their gene expression was assessed at various stages of somatic embryogenesis in *Vitis vinifera*: heart, torpedo, mature embryo, and regenerated plants. It was shown that, at all stages, Put dominated among PAs. In the embryogenic callus, Put content attained 5 mM/g fr. wt; it was represented only by its free form. At later stages of differentiation, including regenerated plants, Put occurred in free and conjugated forms. In all samples, ODC activity exceeded that of ADC. The levels of expression of the ODC and SAMDC genes were correlated with the activities of corresponding enzymes and the levels of Put and Spd at the early stage of embryogenesis. The absence of correlation between the content of free PAs, activities of the enzymes of their biosynthesis, and the levels of gene expression at later stages of embryogenesis and in regenerated plants might result from switching on cell regulatory systems, such as oxidative PA degradation and conjugate formation. It should be noted that, during embryo development with most active cell division, all PAs (Put, Spd, and Spm) were present in increased amounts and in the active free form. It is likely that, at the early stages of somatic embryogenesis, free PAs were critical growth factors involved in the processes of proliferation.

As was mentioned above, Put and Cad belong to PAs controlling cell expansion [4,26,180,185,186]. At the same time, some studies appeared demonstrating that the cells at the tip and marginal regions of the youngest tobacco leaf, which had the highest capacity for auxin-induced growth and the highest auxin levels [190], also had the highest titer of all PAs and the highest biosynthetic activities [11]. It is very interesting that auxin levels in tobacco, which decreased with their aging [190], were negatively correlated with the age-mediated induction of aminooxidase expression in the vascular tissues of tobacco and PAO was down regulated by auxin in the maize mesocotyl [83]. Paschalidis and Roubelakis-Angelakis [84] suggested that the temporal modulation of *PAO* gene expression might be also down-regulated by auxin in tobacco tissues, because the youngest leaves had the lowest DAO and PAO specific activities, and age-induced increase in *PAO* expression in the older leaves could be the result of a complex regulation. It is known that the levels of auxin and other growth-activating phytohormones in plant tissues under stress conditions could be decreased, which excludes the above phenomenon from consideration.

Polyamines and Stress

Polyamines and Oxidative Stress

Under stress conditions, the generation of reactive oxygen species, hydrogen peroxide in particular, is activated in plant cells. Stress-induced oxidative stress is one of the early responses to abiotic factors. Numerous reports appeared about stress-induced accumulation of PAs in various plant species [7]. Only few publications concern PA oxidative degradation under the effect of abiotic factors, although, as was aforementioned, PAs are one of the sources of hydrogen peroxide, which is one of the most widely spread reactive oxygen species even under normal conditions. In this connection, a question arises whether H_2O_2 produced in the reactions of PA catabolism contributes much into damaging effects on plant cells and whether peroxide can be involved in adaptation processes.

Until recently, hydrogen peroxide was often considered only as a toxic metabolite and the cause for programmed cell death [191,192]. In recent years, our notions about peroxide changed from the statement of the fact of its presence in the plant cell to the recognition of its signaling function [193]. Thus, it was established that generation of H_2O_2, a relatively weak oxidizer and a long-living molecule capable of diffusion from the sites of its production to neighboring cells and tissues, could fulfill a signal role in plant adaptation [193]. Plant cells have a rather wide range of peroxide sources: from electron transport chains of chloroplasts and mitochondria to NADPH-oxidase of the plasma membrane; however, these sources differ in their efficiency [194]. PAs are less studied sources of peroxide. Spd and Spm are believed to be most efficient antioxidants, which are considered scavengers of oxyradicals [195-197]. The involvement of PAs in oxyradical scavenging is based on the easy oxygen-dependent autooxidation and enzymatic oxidation of amino groups catalyzed by DAO and PAO and also on the PA capability of accumulation under stress conditions. However, a high level of endogenous PAs and plant tolerance to oxidative stress can be based not only on stress-induced but also on the constitutively high PA biosynthesis. In such plants resistant to oxidative stressors, in particular to paraquat (methylviologen), which breakdown results in the formation of $O^{\cdot}_2$, a high level of constitutive synthesis of both ADC and ODC was found, and the content of PAs was by two to three times higher than in sensitive cultivar [198]. In this case, as it was shown for resistant *Conyza bonariensis* biotype, plant pretreatment with paraquat did not induce the accumulation of Put and Spd but activated antioxidant enzymes. Similar pattern was observed for wheat cultivar displaying cross-reactivity to drought and paraquat and for another species of *Coniza (C. canadensis)* resistant to paraquat. Moreover, only in resistant biotypes, the activities of antioxidant enzymes were high. Treatment of such plants with Put improved further resistance to oxidative stress, but this effect was not observed for a sensitive biotype.

Ye *et al.* [198] consider several possible mechanisms explaining a positive correlation between plant resistance to paraquat, constitutive generation of the high level of PA by corresponding enzymes, and the activities of antioxidant enzymes. Most probable causes are the following: (1) PAs exert their action at the level of genes (genome) by selective inhibition of cytosine DNA methylases and permitting expression of the genes controlling various stages of differentiation [199, 200] or the genes responding to stress [201]; (2) PAs affect gene expression by changing the sequence of specific DNA-protein interactions [202, 203]; and (3)

PAs activate or modulate protein kinases such as CK2 in the signaling pathway [204] and inducing genes for antioxidant enzymes [80,81].

The observed involvement of constitutively high activity of enzymes for PA biosynthesis, especially Put, in plant defense against oxidative stress motivates a study of PA metabolism and their role in naturally tolerant ecological groups of plants such as halophytes, xerophytes, and heavy-metal accumulators.

Among such plants, the common ice plant (*Mesembryanthemum crystallinum* L.) is of interest because it manifests a rather low salt tolerance at early developmental stages, and the halophyte type of its tolerance is developed in adult plants after their transit from C_3- to CAM-photosynthesis [205]. In response to NaCl salinity, only adult plants accumulated a diamine Cad and increased DAO activity. Under short-term treatment of control plants and plants grown under salinity conditions with Cad or Put (1 mM) DAO is activated sharply H_2O_2 content is increased, and guaiacol peroxidase covalently bound to the cell wall is activated. The specific inhibitor of DAO (aminoguanidine) abolished DAO activation and peroxide generation. Electron-microscopic examination demonstrated the formation of a suberin plate outside of the cell wall after treatment of the common ice plant leaves, grown under salinity conditions, with 1 mM Put during 6 days [206, 207]. Cell wall suberinization serves as an additional barrier for ion penetration into the cells under salinity. Acceleration of this process with exogenous Put argues convincingly for the involvement of PAs and the product of their metabolism, hydrogen peroxides, in the development of long-term mechanisms of halophyte adaptation.

PA signaling function in plant defense against oxidative stress is not less important. Thus, it was found that 1 mM Cad added to the nutrient medium for intact common ice plants for 2 h induced transcription of the gene for cytoplasmic Cu/Zn SOD form [81]. The addition of the inhibitor of diamine oxidative degradation, 1 mM AG, along with Cad to nutrient medium did not reduced the level of mRNA, which indicates that non-oxidized diamine affected this gene transcription. Root treatment with 1 mM H_2O_2 increased the level of mRNA as well but to a lesser degree. This supports a previously suggested hypothesis [68] that stress-induced Cad accumulation in the common ice plants and its capability of long-term transport permitted Cad to play a role of a stress signal, which switches on the plant defense mechanism directed, in this case, to the improvement of cell antioxidant activity. In this connection, it should be mentioned that generation of reactive oxygen species by the common plant ice increased sharply during the period of CAM-photosynthesis, which, in its turn, activated antioxidant systems. On the other hand, an enhanced transcription of the genes for antioxidant enzymes did not always correlate with the activity of corresponding enzymes. A possible cause for this discrepancy might be direct effects of peroxide on the activities of peroxide-sensitive enzymes. In the common ice plants, such an enzyme might be Cu/Zn SOD isoform located in the apoplast. We demonstrated that, after the treatment of the common ice plants with low concentrations of Cad and Spm (below 1 mM), PAs behaved as antioxidants, whereas high PA concentrations manifested prooxidant properties due to active formation of peroxide and increased pH (>7.0) in the apoplast. In this case, PAs facilitated the reverse reaction with the formation of superoxide (O_2^-) from H_2O_2 (data not shown). Superoxide radical produced during a burst of PA oxidative degradation could serve a signal for enhanced transcription of the corresponding gene. Switching on such a compensatory mechanism is evidently typical for the functioning of defense systems in stress-resistant plant species.

Products of PA Catabolism and their Physiological Role

The physiological role of some of the products of PA catabolism remains to be elucidated. As was considered above, 1,3-DAP could be used as a donor of amino groups in the biosynthesis of unusual PAs, be accumulated in the free form [54], or be converted into β-alanine. The osmoprotectant β-alanine betaine is produced by alanine methylation; it is required for the osmoregulation in some halophytes, *Limonium tataricum* for example [57,96]. In Arthur Galston laboratory [26], it was shown that 1,3-DAP, occurring in grasses along with usual PAs, could participate in membrane defense against lipid peroxidation, retard senescence like Spd, and suppress protease and ethylene evolution. Since both enzymes of PA oxidative degradation are mainly localized in the apoplast and associated with the cell wall, they are considered H_2O_2-generating systems required for lignification, suberinization, and the formation of cross bridges between the components of the cell wall during plant normal growth and a defensive factor under unfavorable conditions [87].

In order to examine the control of the level of endogenous PAs by their oxidative degradation, *Nicotiana tabacum* plants transformed with constructs containing PAO cDNA from *Zea mays* (*MPAO*) and DAO cDNA from *Pisum sativum* (*PcuAO*) were obtained [208].

These studies showed that both types of transgenic plants (MPAO and PcuAO) produced a great amount of H_2O_2 in the presence of exogenous substrates (Spd and Put). In spite of the fact that both recombinant proteins in tobacco plants were actively synthesized and present in the apoplast, like native proteins in wild-type plants, their activities determined only low PA content in the intercellular space, which was characteristic for both transgenic and wild type plants. High activities of DAO and PAO in transgenic plants reduced the level of endogenous free PAs insignificantly. The amount of H_2O_2 produced in the suspension cells from transgenic tobacco leaves after addition of 1 mM Spd into the culture medium was sufficient for triggering the apoptosis program. In transgenic plants, Spd-induced oxidative stress was clearly transient with a highest development in 30 min after the addition of exogenous PA; it required novel proteins putatively with antioxidant activity. Recently, a capability of PAs to induce expression of antioxidant genes was demonstrated for Spd in the case of tobacco plants [80] and for Cad, for a halophyte *Mesembryanthemum crystallinum* [81]. It is worth mentioning that, in the suspension of transgenic tobacco cells harboring PAO cDNA, the kinetics of H_2O_2 accumulation did not coincide with a gradual reduction in the content of Spd in the cells. This fact indicates the involvement of PA excretion into the culture medium into the control of the PA pool in the cell.

These studies with transgenic plants proved experimentally for the first time that modulations in the level of endogenous PAs only slightly depended on their oxidative degradation under normal physiological conditions, which indicates the occurrence of compensatory mechanisms maintaining PA homeostasis in the cells.

Conjugates of Polyamines and their Role Under Stress Conditions

According to some researchers [8], free PAs, Put in particular, comprise from 50 to 90% of their total content in the cell. The smaller PA part is bound with low molecular weight and high molecular weight molecules [7,12].

The formation of PA conjugates based on posttranslational covalent binding with proteins

is catalyzed by specific enzymes, Ca^{+2}-dependent and Ca^{+2}-independent transglutaminases (EC 2.3.2.13), which are localized both inside the cells and in the intercellular space [209]. It was reported that transglutaminases could be activated under stress conditions. Some authors consider the γ-glutamyl derivatives of PAs, which bridge two polypeptides, as modulators of enzyme activities or structural proteins [209,210].

Plant physiologists engaged in stress studying pay a great attention to PA conjugates with hydroxycinnamic acids. In this case, PAs produce amide bonds using CoA esters for activation of carboxylic groups with the help of enzymes known as transferases [12,211]. Such PA conjugates were found in many plant families. Put produces a monomer form (acid-soluble fraction) with cinnamic, coffeic, and ferulic acids or dimer form with hydroxycinnamic acids (acid-insoluble fraction), whereas Spd produce dimers or three-substituted conjugates. These conjugates are important for the control of the intracellular PA concentrations [111] and for their interaction with components of the cell wall, especially hemicelluloses and lignin [212]. Slocum and Galston [119] suppose that the exchange between free and conjugated PAs in the plant cell is limited. Other researchers [96] believe that PA conjugates with hydroxycinnamic acids could regulate the intracellular PA pool, serve for PA transport or even be a substrate for aminooxidases and peroxidases [122, 213, 214]. Most important property of PA conjugates with phenolic acids for plant adaptation to stress conditions is their antioxidant activity. Antioxidant properties of conjugated PAs were first noted by Bors *et al.* [196]. According to their study, PA conjugates with coffeic, cinnamic, and ferulic acids displayed a higher constant of binding to reactive oxygen species than free PAs, which contrasted to early notion about an important role of free PAs as radical scavengers [195].

This means that plants experiencing stress should produce conjugated PAs to contradict damaging effects. However, published data about the content of soluble PA conjugates in stressed plants are rather contradictory [104, 215-217], which could be determined by species-specificity and the content of phenolic compounds and PAs as substrates for the production of acid-soluble PA conjugates. In some studies [218-221], it was shown that jasmonates stimulated the production of plant secondary metabolites, including hydroxycinnamic acid and PAs. Thus, in barley leaves, the level of free Put, Spd, and Spm and their conjugates increased markedly on the 4th day after treatment with methyl jasmonate, which was accompanied by leaf increased resistance to powdery mildew [216]. It is of interest that, in this study, DAO activation was observed along with the methyl jasmonate-induced increased levels of PAs and their conjugates, which could be a response to the increased level of free Put. At the same time, a coordinated increase in the activity of peroxidase in response to the increased level of phenolic compounds, required for its functioning in the apoplast, abolished a possible toxicity of H_2O_2, the product of DAO activity.

Polyamines and stress ethylene

A typical example of multifunctional interactions in plants under stress conditions is those between PAs of Put family (Spd and Spm) and ethylene. They have a common precursor SAM for their biosyntheses [1, 222, 223]. Along with a competition for a common precursor, other interactions could arise between PAs and ethylene under stress conditions; they are manifested in mutual inhibition of their biosyntheses, which became a basis for the notion about competition

between major PAs and ethylene [26]. Such an interaction takes an important place in the coordination of physiological processes because PAs and ethylene exert often opposite effects. For example, Spd retards senescence, whereas ethylene accelerates it [26].

To understand SAM functional role as an intermediate in PA and ethylene biosyntheses, we should take into account the following points: (1) SAM is actively used in plant cells as a main donor for transmethylation of proteins, nucleic acids, polysaccharides, and fatty acids [224] and (2) 5-methylthioadosine (MTA), a byproduct of SAM degradation during synthesis of Spd, Spm, and 1-aminocyclopropan-1-carboxylic acid (ACC), can be recycled by MTA nucleosidase into methionine and further into SAM [225, 226], *i.e.*, SAM is positioned on the cross of many metabolic pathways both in plants and animals. To assess SAM role in the biosynthesis of major PAs (Spd and Spm), it should be kept in mind that, in stressed plants, the pool of SAM could increase due to stress-induced accumulation of S-adenosylmethionine synthase (SAMS) transcripts [40], i.e., under stress conditions, SAM homeostasis is maintained to increase plant adaptive potential.

Some researchers demonstrated that interaction between PAs and ethylene could not be limited only by their antagonism. Thus, pea seedlings responded to ethylene treatment by a reduced activity of ADC and increased activity of LDC and increased content of Cad [227, 228]. The stimulatory effect of ethylene on Cad biosynthesis did not attract attention for a long time, although processes of their biosyntheses are indirectly interconnected because Cad is formed in the side branch of the aspartate pathway resulting in biosynthesis of methionine and SAM [63]. Moreover, SAM is required for the formation of ACC, a precursor of ethylene.

A facultative halophyte *Mesembryanthemum crystallinum* turned out to be a very convenient model for investigating the interaction between Cad and ethylene under stress conditions. In this plant, aspartate, a distant precursor of lysine, is one of the main metabolites produced from oxalacetic acid during CO_2 assimilation in CAM-photosynthesis. It was demonstrated that, in the common ice plants, stress-induced Cad accumulation coincided with the developmental stage when plants transited from C_3- to CAM-photosynthesis [67,68]. In this period, the common ice plants responded to heat shock (HS) by a transient ethylene evolution and a subsequent interorgan translocation of Cad. Under NaCl salinity, the level of endogenous ethylene in plants increased and Cad accumulated in the leaves. To confirm a possible relation of HS-induced Cad translocation from the leaves to the roots to transient ethylene evolution, two lines of *Arabidopsis thaliana* were used as model plants: wild type (Col-0) and a mutant (*ein4*) displaying disturbed ethylene reception. It was established that HS-induced interorgan translocation of Cad, as distinct from Put and Spd, was related to the functioning of the ethylene reception system. Ethylene-dependent Cad formation was proven by this diamine accumulation in detached leaves of the common ice plant exposed to the atmosphere of ethylene or incubated in the presence of its precursor, ACC [68, 229]. The phenomenon of ethylene-dependent Cad accumulation permitted a study of putative mechanisms of hormonal signal transduction, which were not examined until recently. It was supposed that protein phosphorylation was involved in ethylene signal transduction, like in other signaling pathways. Some researchers [230, 231] demonstrated that plant treatment with ethylene enhanced phosphorylation of proteins, the components of the signal transduction pathway. In order to elucidate whether the processes of protein phosphorylation/dephosphorylation are involved in the ethylene-induced Cad production, various inhibitors of protein kinases and protein phosphatases were used: Sodium vanadate,

the inhibitor of tyrosine phosphatases [231], NaF, the blocker of membrane phosphatases [113], and apigenin, the inhibitor of some MAP kinases [232]. The effect of inhibitors on Cad formation in detached leaves of the common ice plant was assessed by the level of LDC [229]. All inhibitors tested abolished a stimulatory effect of ethylene on the LDC activity, and this was first unambiguous proof of the involvement of protein phosphorylation/dephosphorylation in the ethylene-induced Cad formation in plants (Figure 2).

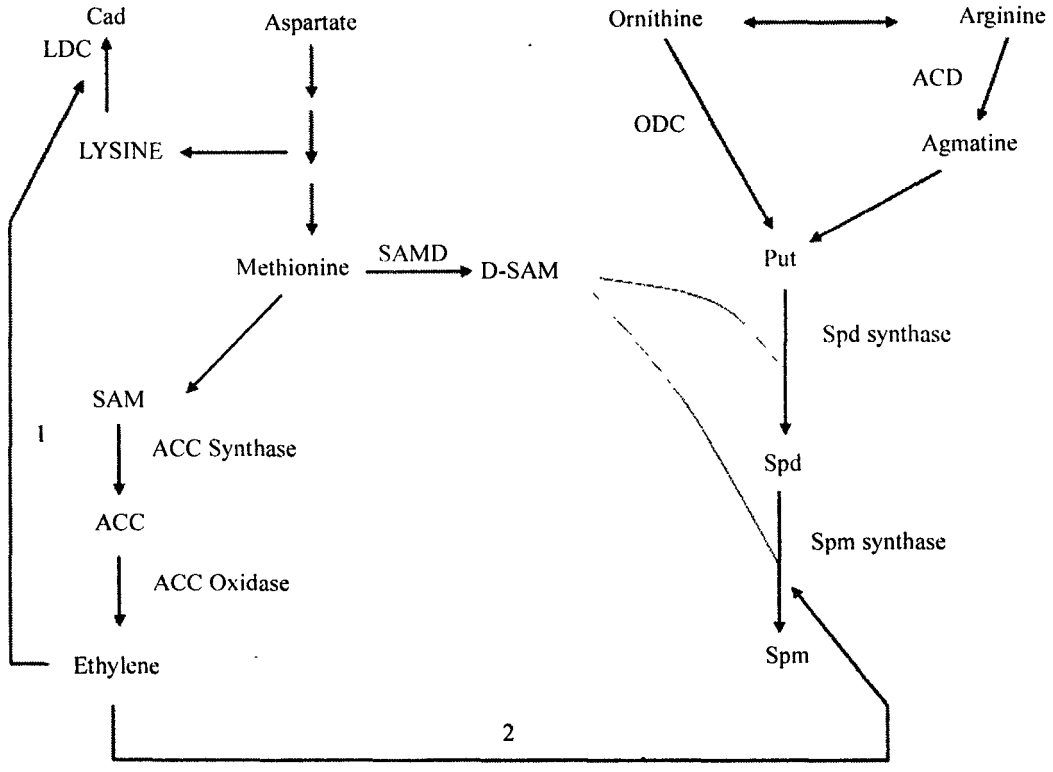

Figure 2. Polyamine biosynthesis in plants and its possible regulation by ethylene (1) activation; (2) inhibition.

It is evident from the results obtained that, along with competitive interrelations between major PAs (Spd and Spm) and ethylene, which could be manifested under stress conditions, the interaction between Cad and ethylene may be rather evaluated as synergistic. The phenomenon observed permits a fresh insight into the problem of compensatory reactions maintaining PA homeostasis required for plant survival under stress conditions. However, Cad accumulation and an increased level of endogenous ethylene in the common ice plants in the period of CAM-photosynthesis functioning did not affect expression of the gene encoding a key enzyme of CAM metabolism, PEPC [233]. Cad accumulation occurred at the later stages of the common ice plant development and was not associated with CAM induction providing for plant adaptation to water deficit. The mechanism of the common ice plant adaptation to salt stress related to Cad accumulation might be as follows: the high Cad concentrations retar . cell

wall expansion due to its suberinization and lignification, thus reducing cell wall permeability for salts. This conclusion arises from the analysis of phenotypical responses of the common ice plant seedlings to various concentrations of exogenous Cad and from electron-microscopic examination of the cell wall structure [206, 207].

Protective and Regulatory Role of Polyamines

Most widely accepted and experimentally proved view is that PAs exert their protective action due to their chemical structure, as polycations. This is largely determined by the shift of their electron density toward nitrogen atoms under physiological pH values; therefore, PAs behave as bases (pK = 9-11). This explains the readiness of PA electrostatic interaction with negatively charged phosphate groups of phospholipids and nucleic acids and with carboxylic groups of proteins and also the PA capability of covalent binding with proteins at the stage of their posttranslational modification [4, 7, 26, 119].

Such defensive properties are ascribed primarily to high-molecular-weight PAs (Spd and Spm) and unusual multipolyamines with longer and often branched molecules, which efficiency is directly related to the increased number of amino groups in their molecules. Unusual PAs (norspermidin and norspermin) were found in such plants as *Canavalia gladiata, Vicia radiata,* and *Vicia sativa* [52, 53] and in the cultured calluses of thermotolerant rice cultivar after a short-term HS (45°C, 30 min) [54]. Plant adaptation to abiotic stresses associated with Spd and Spm accumulation might be largely depend on enhanced activity of the key and rate-limiting enzyme, SAMDC. In some systems, Spd and Spm accumulation was correlated with improved plant tolerance to salinity and low temperature [20, 21, 234, 235]. In transgenic arabidopsis, an increased SAMDC expression and Spd and Spm accumulation improved plant tolerance to chilling and salinity [73]. Overexpression of Spd synthase cDNA from *Cucurbita ficifolia* in *Arabidopsis thaliana* significantly increased Spd level and, consequently, enhanced tolerance to various stresses [236].

PA binding to proteins or nucleic acids not only protects them from degradation but also provides a molecule the most stable conformation under stress conditions. Thus, Spd and Spm retard cell aging, which is accelerated under stress conditions, due to suppression of the enzymes degrading biopolymers (DNases, RNases, and proteases) and prevent chlorophyll breakdown [237]. Exogenous application of Spd stabilized a native structure of thylakoid proteins D1 and D2, cytochromes, and also a key photosynthetic enzyme Rubisco in oat plants subjected to osmotic stress [14,36].

All PAs are capable of binding to A- and B-DNA: in A-DNA, binding occurs mainly to the major groove, whereas in B-DNA, Put and Cad bind to sugar-phosphate backbone and Spd and Spm, which contain more amino groups, bind to both sugar-phosphate backbone and major and minor grooves [238]. Experiments with B-DNA differing in the guanine to cytosine ratio showed that high molecular weight PAs interacted mainly with phosphate groups and did not affect a native secondary structure of DNA, thus providing for normal transcription of stress-induced genes. Such interaction was evidently unspecific and did not almost depend on DNA nucleotide sequence [239]. PAs could inhibit DNA methylation, which permits expression of specific genes responsible for the synthesis of stress proteins [199, 200]. Spm and to a lesser degree Spd are capable of shifting a dynamic equilibrium between B- and Z-DNA and are

involved in DNA spiralization [240]. Earlier studies indicate that PAs are capable of complex production not only with DNA but also with RNA and ribosomes [241, 242]. PA protective role is manifested in their capability of neutralizing the action of reactive oxygen species dangerous for the cell structures and accumulated under the effect of various abiotic and biotic stresses [81, 195-197].

Recently, it was found that PAs could substantially affect the conductivity of ionic channels in plants. Thus, Put, Spd, and Spm blocked fast and slow vacuolar channels, including calcium channels, and the effect was proportional to PA charge ($Spm^{+4} > Spd^{+3} > Put^{+2}$) [167]. The capability of biogenic amines to affect stomatal conductivity under stress conditions was also connected with their charges. It was shown that this universal for plants physiological response to stress was based on the PA-induced blockage of potassium channels in the plasma membrane of guard cells, which increased their turgor and, as a consequence, resulted in decreasing the stomatal aperture. In particular, PAs blocked potassium channel in the plasma membrane into the mesophyll cells harboring the *KAT1* gene encoding one of such channels. It is of interest that, in spite of induction of one and the same response by PAs and ABA, the underlying mechanisms are different because ABA inhibits inward potassium channels. PAs also affected stomata closure when penetrated into the cytosol, implying the presence of an intermediate cytoplasmic factor involved in the induction of this response [243]. PA control of ionic channels might be adaptive under stress conditions. Thus, potassium channels are efficient regulators of cell stimulation and a major target for extracellular and intracellular factors. Blocking potassium channels with Spd was shown to be a major impulse permitting for adaptation of cell stimulation in response to numerous biological stimuli [244]. PA accumulation in plants subjected to osmotic stress could be required for transduction of the osmotic signal [13]. PAs were shown to suppress plant responses to osmotic stress [245].

The regulatory role of PAs manifesting in the activation of protein and nucleic acid syntheses was demonstrated in both prokaryotes and eukaryotes [7]. The involvement of PAs in the signal transduction in plants was detected only in some cases [170,246], whereas in animals and bacteria, it was reliably shown [166,247]. In addition, PAs were found to activate protein phosphorylation and the activities of definite protein kinases [114,248,249].

The abundance of data concerning stress-dependent PA accumulation in plants raises a question about their role in adaptation [7]. Plant cell metabolism is changed to prevent damaging consequences of stressor action [250]. This is attained by realization of two pathways of living organism adaptation to extreme factors operating simultaneously or successively: (1) induction of the synthesis of new macromolecules with new properties, which provide for a normal proceeding of the cell metabolism under stress conditions and (2) optimization of the intracellular medium for functioning of the enzymic systems due to the accumulation of low molecular weight organic compounds with protective and/or osmoregulatory properties. Both pathways of adaptation are directed to solving the same tasks, namely, organism providing with energy, reductants, precursors of nucleic acids and proteins, and also to the maintenance of cell regulatory system functioning under stress conditions.

Since the time when an active Put accumulation was observed in plants in response to K^+ deficit [251], salt shock [252], water deficit, and low pH, the investigations of stress-induced changes in PA metabolism and their role in plant responses to abiotic factors remain to be one of key problems of plant adaptive strategy [6].

However, in spite of a large progress in the elucidation of mechanisms of PA anabolism and catabolism in the plant cell, a general scheme of controlling the PA endogenous level under stress conditions is not yet suggested.

Recent elucidation of the mechanisms of transcriptional, translational, and posttranslational regulation of PA biosynthesis in plants permitted us to present a hypothetic model of the control of PA intracellular content and their physiological role under stress conditions. We based on the data obtained in some studies that the stress-induced accumulation of Put is characteristic of stress-sensitive plants and Spd and Spm accumulation, of tolerant plants.

Considering the sum of published data, we can state that stressors induce a transient accumulation of free PAs in plants during first minutes and hours of stress; thereafter, days are necessary to maintain PA homeostasis in the cells at the level required for the development of long-term plant adaptation to stress. The time course of changes in PA metabolism in the plant cell can be described as a primary response to rapid disturbances dangerous for plant life: turgor loss and generation of reactive oxygen species. These events activate the signaling cascades inducing a transient Put synthesis in stress-sensitive plant species. In stress-tolerant species, the Spd and Spm levels required for long-term plant adaptation to stress is maintained constitutively by high activities of the genes encoding enzymes of their biosynthesis [236, 253]. The level of Put decreases because of its consumption as a precursor in these syntheses. In Cad-containing stress-tolerant plant species, increased levels of Spm and Cad are maintained, compensating a reduced level of Put in the cells.

Conclusions and Future Perspectives

Considerable evidence indicates that PAs are involved in a wide range of plant processes, including adaptation to abiotic stresses. However, their precise role in these specific processes remains to be established. The PA biosynthesis pathways are ubiquitous in living organisms and include a limited number of enzymes involved. Thus, the PA biosynthesis pathway represents an excellent model to test the hypotheses of PA involving in plant protection against stresses.

In recent years, various approaches have been developed to manipulate PA metabolism: specific inhibitors, mutants, and transgenic plants [5]. Taken together, some results about elevated levels of Spd and Spm in stress-tolerant plant species suggest that the levels of these PAs in the cells are under a strict homeostatic regulation due to a supramolecular organization of some enzymes of their biosynthesis. Application of advanced genomic and proteomic approaches will help to elucidate the role of PAs in particular plant processes in stress tolerance [6].

Much of the recent advances in understanding the role of PAs in plant response to abiotic stress came from studies that employed stress-tolerant plant genotypes, for example halophytes, in comparison with stress-sensitive plants. Halophytes might evolve unique mechanisms of their responses to stress, which were not found in glycophytes. The common ice plant (*Mesembryanthemum crystallinum*) and other halophytes were widely used to further understand the PA involvement in increasing plant tolerance. Regretfully, while a large expressed sequence tag database has been amassed for the common ice plant, as well as the mutant collection was initiated [254, 255], all attempts to transform this plant were unsuccessful.

Recently, several groups of researchers have reported the usage of a close relative of *Arabidopsis*, salt cress (*Thellungiella halophila*), with a genome size approximately twice that of Arabidopsis as an appropriate halophytic model [256, 257]. This plant is extremely tolerant to cold, drought, and salinity. Genomic tools in place and being created will amplify its potential as an experimental system, permitting for a discovery of stress-induced genes and related genes of PA metabolism.

One of the obstacles in understanding PA biological role is scarce information about the cellular and subcellular localization of PAs and their biosynthetic enzymes in plants, especially under stress conditions. There is a gap in our knowledge of the translocation of free PAs and their interaction with hormones, of their role in gene expression, as well as of the role of bound PAs in plant stress tolerance.

In general, the use of molecular approaches, cloning of genes for PA biosynthetic enzymes in particular, production of transgenic plants, isolation and characterization of mutants defective in PA biosynthesis will provide a better understanding of the PA role in plant adaptation to stress conditions. The improvement of crop tolerance to abiotic stresses by cellular and molecular modifications of PA metabolism is in progress. However, a thorough comparative study of the expression and function of members of the PAs gene families in extreme halophytes and xerophytes will eventually assist in the breeding of stress-tolerant crop plants.

Acknowledgements

The authors are grateful to Professor Nella L. Klyachko of Institute of Plant Physiology (Moscow, Russia) for valuable assistance in translating this manuscript from Russian into English. This work was partially supported by the Russian Foundation for Basic Research (Project Nos. 04-04-48392, 04-04-49589) and by the program of the Presidium of Russian Academy of Sciences (Molecular and Cell Biology).

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7 Response of Plants to Salt and Water Stress and the Roles of Aquaporins

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Introduction

Water is the major component of most cells and plays a vital role in the survival of all living organisms on earth from mammals and plants down to microscopic life. A fundamental part of the role of water in life is its involvement in movement of molecules from one location to another within and between cells and tissues. Plants are immobile and often exist in challenging environments; therefore, they rely largely on supply of water from the soil for their growth and development. A number of abiotic stress factors related to plant-water relations, such as drought, salinity, chilling, frost and flooding, negatively affect the overall growth of plants, through variously affecting root function, slower growth rates leading to stunted form, metabolic changes, reduced yields, reduced germination and even plant death in extreme conditions [1,2]. These issues become important especially as the expected rise in global temperatures suggests there will be no alleviation to such problems and as the world population constantly increases, and a clearer understanding and improvement of plant tolerance to these stresses is needed, particularly in important crop plants such as rice and wheat.

It was believed until recently that water travelled through biological membranes simply by osmosis; however, it has become clear in the past few years that membrane embedded water channel proteins called *aquaporins* act as specific pores for water and are responsible for its selective transport. Although the presence of water channel proteins was predicted some time ago [3,4], the first such protein was only discovered recently when the water permeability function of 'CHIP28' (now known as AQP1, aquaporin-1) was discovered through cell swelling assays utilising the *Xenopus laevis* oocyte expression system [5]. Water flows through living tissues in plants as cells lose water as a result of transpiration and the expansion of growing cells. Of the two major routes of water transport within and between plant tissues, the apoplastic route appears to be driven by physical forces and regulated mostly by differences in water

potentials between the soil, plant and atmosphere [6]. However, the hydrophobic Casparian strip in the endodermis prevents water from continuing entirely along the apoplastic route and forces water molecules to enter the symplastic route, and it now appears that this route may be regulated largely by the aquaporins [7]. Previous studies have focussed on other genetic factors involved in salt or water stress tolerance, e.g., on a region of the D genome involved in salt-tolerance of the bread wheat (*T. aestivum*) compared [8] to that of the durum wheat (*T. turgidum*), or the sodium exclusion locus in wheat [9], or genes of pathways involving synthesis of proline, a widely distributed osmolyte with a role in osmotic adjustment for tolerance to water deficit [10], or trehalose, reported to improve desiccation tolerance [11]. However, plant resistance to these environmental stresses possibly involves combined effects of multiple genes, and the aquaporins make excellent candidates for such studies, especially as these are the only cellular membrane proteins known with specific water-conducting ability. Some key observations in recent years regarding the regulation of expression of these genes and their possible roles in environmental stress response are the focus of this chapter.

Typical Structure and Transport Roles of Aquaporins

Aquaporins are 26-30 kDa proteins and belong to the large MIP (major intrinsic protein) superfamily and appear to have evolved very early during evolution, as they have now been identified in a wide range of organisms including plants [12], humans [5], yeast [13], bacteria [14], and archaea [15]. The amino acid sequences of MIP members are highly conserved, with most MIPs characteristically containing two copies of the MIP signature sequence known as the NPA (Asn-Pro-Ala) motif and consisting of six transmembrane alpha helical domains (TMHs) linked by five inter-helical loops with the amine and carboxyl termini orientated into the cytoplasm (Figure 1). Each half of the protein is obversely symmetrical and the two halves combine to produce the 'hour glass model' structure [16]. Loops B and E are hydrophobic in nature and are themselves small helical regions that dip into the membrane. Each of these usually contains an NPA motif that meets in center of the membrane forming the water transporting pore and providing rapid, single-file water molecule transport in either direction. Of the various subfamilies of MIPs (discussed below), the 'aquaporins' are selective for water and transport water molecules through biological membranes with minimal energy expenditure [17]. The selectivity of aquaporin channels is due to re-orientation of water molecules during transport, with the molecules being oriented in opposite directions in the two halves of the channel (Figure 1). This is due to the positive ends of the helix dipoles of the pore causing the water dipole to align perpendicular to the channel axis, and the breaking of hydrogen bonds between neighbouring water molecules by the Asn residue of each NPA motif [17]. Other members of the MIP superfamily include glycerol, urea and ammonia transporters [18-20]. A number of residues within the amino acid sequence of MIPs are considered important for substrate selectivity, including the ar/R (aromatic/arginine) region that forms a tetrad of four residues from TMH 2, TMH 5, and interhelical loop E. This combination of residues exists in the channel pore and is thought to be the primary selective filter of MIPs [21, 22]. For example, substitutions of these residues causes enlargement of the pore aperture and changes in polarity, to accommodate the bulkier, non-polar glycerol molecule [22, 23]. Only the water-transporting MIPs in plants are of relevance to plant-water relations and hence discussed below.

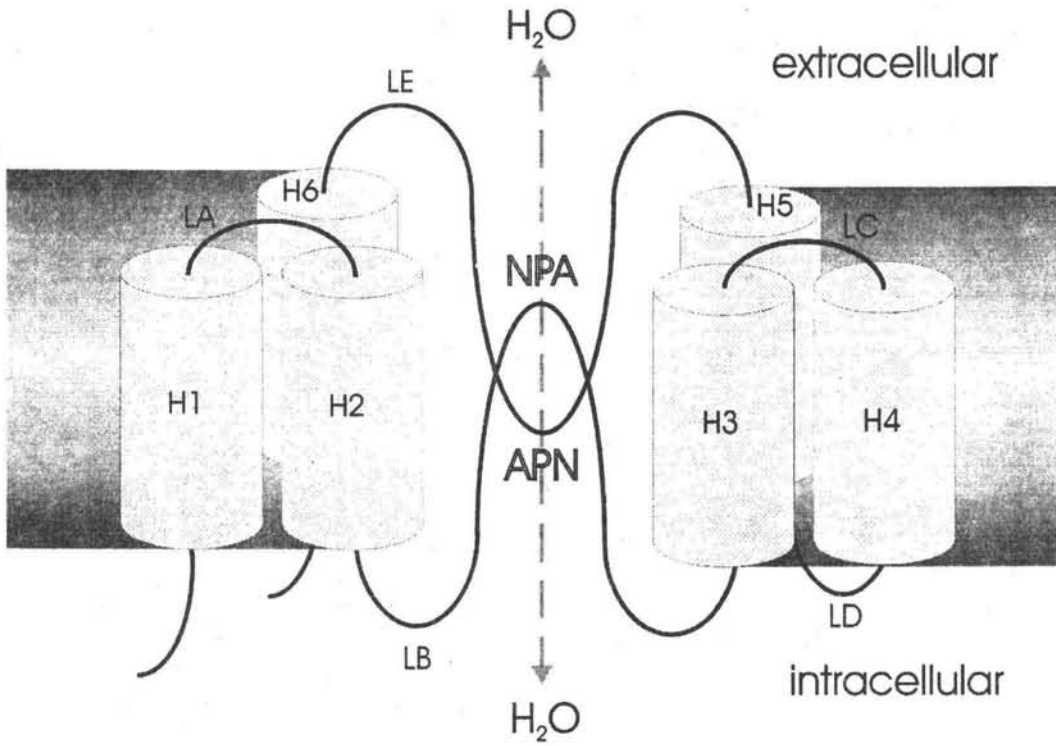

Figure 1. 'Hour-glass' structure of AQP1. H1-H6 indicate the six trans-membrane helices and LA-LE indicate the five interconnecting loops of the conserved structure of aquaporins. The two NPA motifs meet in the center of the membrane, contributing to the water-selective pore region. Figure redrawn, based on Jung et al. [16].

Diversity and Transport Functions of Plant MIPs

Plant genomes appear to encode a larger number of MIP genes compared to other species; for example, thirty five MIPs have been identified in *Arabidopsis thaliana* [24,25] and thirty-one in *Zea mays* [26], compared to only eleven in humans [27]. Plant MIPs are also more diverse in their sequences and are comprised of four major subfamilies; PIPs (plasma membrane intrinsic proteins), TIPs (tonoplast intrinsic proteins), NIPs (Nodulin 26-like intrinsic proteins) and SIPs (small, basic intrinsic proteins). The amino acid sequences of members within each subfamily are conserved, especially within the TMH domains and NPA regions than at the termini which are more variable. PIPs typically have a longer N-terminal sequence compared to TIPs, whilst within the PIP subfamily, the PIP1 and PIP2 members mainly differ in the N-termini, PIP1 members being more extended and also having a shorter C-terminal region than PIP2 [28]. Unlike the vast majority of plant MIPs, some members of the NIP and SIP subfamilies only contain one NPA motif [29]. The diversity and complexity of plant MIP genes is perhaps not surprising, given their immobile state and the absence of a circulatory system, and suggests that these genes may have important and varied roles.

Plant MIPs have been shown to exhibit a range of expression patterns, often specific to the genes, tissues or physiological conditions, further suggesting specialised functions for at least some members of this superfamily. In some cases, many members of the same subfamily may be expressed simultaneously in the same tissue, for example PIP1a, PIP1b, PIP1c, PIP2a and PIP2b are all expressed in the roots [30], or, in contrast, the expression of some members appears to be tissue specific, e.g., the TobRB7 transcripts of tobacco are absent in shoot, leaf and stem tissue but detected specifically in root tissue, in particular in the meristem and immature central cylinder regions [31]. In terms of functional specialisation, the PIP and TIP members appear to be the major water transporters, with both subfamilies showing high rates of water permeability [12,32]. Of particular interest is the fact that members of the PIP subfamily appear to play a particularly important role in controlling transcellular water transport, as evidenced by a transgenic *A. thaliana* plant expressing double antisense *PIP2* and *PIP1* isoforms showing a decrease in osmotic hydraulic conductivity in root and leaf protoplasts [33] and a gene knockout of *PIP2;2* showing similar results in root tissue [34]. It has also been observed that members of the PIP2 subfamily show greater water permeability properties than those of the PIP1 subfamily and hence probably possess different functions [35]. Some TIPs have also shown a high degree of water permeability function [12], and the RNA(i)-targeting of TIP1;1 in *A. thaliana* resulting in plant death [36] demonstrates that TIPs are also vital to plant survival.

Aquaporins and Stress Response: General Trends and Tools of Study

Due to the PIP and TIP members being the major water transporters, it is not surprising that these two subfamilies have been targeted for studies on stress response over the lesser characterised NIP and SIP subfamilies. Regulation of aquaporin transcripts has been noted in response to a number of abiotic stresses that depend on plant-water relations and water transport, i.e., drought, salinity, chilling, and flooding, in a variety of plant species. Situations such as drought and salinity require a change in plant water status in order to survive through such stresses. Salinity and water stress appear irrevocably linked because high concentrations of external Na^+ and Cl^- leads to a lower extra-cellular water potential and thus water deficit. In the laboratory, modelling of salt stress typically involves growing plants in media containing 150 mM NaCl, whereas growth in 250 mM mannitol (or other non-permeable molecule) or abstinence from watering have been used to study response to water stress. The effects of such stresses have been measured by targeting of specific MIP genes of interest using methods such as northern blotting or RT-PCR (reverse-transcription polymerase chain reaction), production of transgenic plants expressing a specific transgene, expression of an anti-sense copy to down-regulate the expression of a native gene, or analysis of gene promoters. Alternatively, 'shoot-in-the-dark' methods have also been used for identifying a large number of stress-responsive genes simultaneously, e.g., through use of microarrays [37]. Both approaches have identified a number of PIP or TIP genes as being responsive to abiotic stress (summarised in Table 1 and 2), and changes have been observed in aquaporin transcript and/or protein abundance. These types of observations indicate that MIP genes may be involved in altering the water status and possibly membrane permeability, by regulation of protein activity and/or abundance in the membrane. Upregulation of MIPs may trigger greater osmotic water permeability to facilitate water flux, whereas downregulation may allow cells to conserve water, thus allowing e‘ icient

Table 1. Summary of MIP regulation in response to salt stress

Plant, MIP gene/isoform	*Effect on transcript and/or protein*	*Reference*
MIPs		
A. thaliana		
At-NLM1	Transcript downregulated	[42]
O. sativa rMIP1 (NLM1/2)	Transcript stable	[43]
PIPs		
A. thaliana		
AthH2 (PIP1b)	Transcript overexpression in tobacco led to better resistance to salinity	[44]
AtPIP1;1	Transcript upregulated in aerial and root tissue	
AtPIP1;2	Transcript upregulated in root tissue	
AtPIP1;5	Transcript downregulated in aerial and root tissue	[40]
AtRD28 (PIP2)	Transcript upregulated	[42]
AtPIP2;2,	Transcript upregulated in aerial	
AtPIP2;3,	tissue	[40]
AtPIP2;6	Transcript downregulated in aerial tissue	
AtPIP2;7,	Transcript upregulated in aerial and	
AtPIP2;8	root tissue	[45]
AtPIP2;3	Transcript upregulated	
C. plantagineum		[41]
CpPIPa2	Transcript downregulated	
H. vulgare		
HvPIP1;3,	Little effect on transcript	[39]
HvPIP1;5	Transcript upregulated in shoots,	
HvPIP2;1	downregulated in roots	
L. esculentum		[46]
TRAMP (PIP1)	Transcript induced	
M. crystallinum	Transcript downregulated, no change	[47,48]
MIPA (PIP1)	in protein	
MIPB (PIP1)	Transcript stable, no change	
MIPC (PIP2)	in protein	
	Transcript downregulated, protein unregulated with lower salt	
N. excelsior		
NeMIP1 (PIP1),	Transcript upregulated	[49]
NeMIP2 (PIP1),		
NeMIP3 (PIP1)		
O. sativa		
WCP-1 (PIP1),	Transcript upregulated in salt	[37]

WCP-1 (PIP2).	tolerant variety	
WCP-1 (PIP1),	Transcript downregulated initially	
WCP-1 (PIP2).	after salt stress but upregulated later, in salt tolerant variety	
RWC1 (PIP1)	Transcript downregulated in root	[50]
R. sativus L.		
RsPIP1-1	Transcript slightly upregulated in hypocotyls	[51]
RsPIP1-2,	No change in both transcript and protein	
RsPIP1-3	Both transcript and protein up-regulated	
RsPIP2-1		
TIPs		
A. thaliana		
At-αTIP	Transcript upregulated	[42]
At-δTIP	Transcript stable	
M. crystallinum		
MIPF (γ-TIP1/2)	Protein down-regulated in shoot and root	[47]
O. sativa		
rTIP1 (γ-TIP)	Transcript upregulated in shoots and roots	[43]
R. sativus L.		
RsTIP1-1,	No change in both transcript and	[51]
RsTIP2-1	protein	
Z. mays CHEM8 (γ-TIP)	Transcript upregulated	[53]

Table 2. Summary of MIP regulation in response to water stress

Plant, MIP gene/isoform	*Effect on transcript and/or protein*	*Reference*
MIPs		
A. thaliana AtSIP1;1	Transcript stable	[54]
At-NLM1	Transcript downregulated	[42]
O. sativa rMIP1 (NLM1/2)	Transcript stable by water stress	[43]
PIPs		
A. thaliana		
PIP1c, PIP1a	Transcript stable during water stress	[30]
AtPIP1;2	dAS (with *AtPIP2;*3) increased root growth and impaired recovery	[33]
AtPIP1;3, AtPIP1;4,	Transcript downregulated by drought	[54]

AtPIP1;5		
AtPIP1;3, AtPIP1;4	Transcript upregulated by water stress in aerial and root tissue	[40]
AtPIP1;5	Transcript downregulated by drought in aerial and root tissue	
AtPIP2;2, AtPIP2;3; AtPIP2;4 AtPIP2;5	Transcripts downregulated by drought Transcripts upregulated by drought	[54]
AtPIP2;6	No effect on transcript levels	
AtPIP2;1, AtPIP2;5	Transcript upregulated by water stress in aerial and root tissue	[40]
AtPIP2;2; AtPIP2;3, AtPIP2;4	Transcript downregulated by water stress in aerial and root tissue Transcript downregulated by water	
AtPIP2;6	stress in aerial parts of plant, only slightly in roots	
RD28 (PIP2)	Transcripts upregulated by dehydration, whole plant, protein induced by dessication	[55]
AtRD28 (PIP2)	Transcripts downregulated by osmotic stress and drought	[42]
RD28 (PIP2)	Proteins stable by dehydration	[32]
PIP2a, PIP2b	Transcript stable by water stress	[30]
AtPIP2;3	dAS (with *AtPIP1;2*) plant showed increased root growth and impaired recovery	[33]
B. napus		
BnPIP1	Transcript overexpression led to increased tolerance to water stress, expression of antisense reduced tolerance to water stress	[5
B. oleracea		
MIPa (PIP1b)	Transcript upregulated by drought	[57]
MIPb (PIP1b)	Transcript stable during drought	
C. plantagineum		
CpPIPa2	Transcript upregulated by dehydration	[41]
CpPIPa2 (PIP1), CpPIPa6 (PIP1), CpPIPa7 (PIP1), CpPIPc (PIP3).	Transcript upregulated by drought	
CpPIPb (PIP1)	Transcript stable by drought	[58]
L. esculentum		
TRAMP (PIP1)	Transcript upregulated by drought	[46]
N. excelsior		
NeMIP1, NeMIP2, NeMIP3	Transcript upregulated by drought	[49]

N. glauca		
NgMIP4	Transcript downregulated by drought	[59]
O. sativa		
OsPIP2a (PIP3/3b), OsPIP1a	Transcript downregulated by drought	[60]
RWC1 (PIP1)	Transcript downregulated by water stress	[50]
RWC3	Transcript and protein upregulated in drought tolerant species, transcript stable and protein downregulated in drought-sensitive species	[52]
R. sativus L.	Transcript and protein stable	
RsPIP1-1, RsPIP1-2, RsPIP1-3	Transcript upregulated within 1 hour in roots, protein downregulated in roots	[51]
RsPIP2-1	Transcript upregulated	
RsPIP2-2 RsPIP2-3	Transcript upregulated within 1 hour in roots	
TIPs		
A. thaliana		
AtTIP1;1, AtTIP1;2 AtTIP2;1, AtTIP2;2.	Transcript downregulated by drought	[54]
δ-TIP, γ-TIP	Transcripts downregulated by osmotic stress, stable during drought	[42]
B. oleracea		
BobTIP26-1 (γ-TIP), BobTIP26-2 (γ-TIP)	Transcript upregulated by drought	[61,62]
C. plantagineum		
CpTIP	Transcript downregulated by dehydration	[58]
H. annus		
SunTIP7 (δ-TIP), SunTIP20 (δ-TIP).	Transcript upregulated by drought	[63,64]
SunTIP18 (δ-TIP), SunRB7 (δ-TIP2)	Transcript downregulated by drought	[64]
Sung-TIP (γ-TIP)	Transcript stable by drought	
N. glauca		
NgMIP2, NgMIP3	Transcript downregulated by drought	[59]
O. sativa		
rTIP1	Transcript upregulated in roots and shoots	[43]
R. sativus L.		
RsTIP1-1, RsTIP2-1	Transcript and protein stable	[51]

water transport [38]. It has also been hypothesised that upregulation of MIP transcripts or proteins may result in the rapid uptake of water into cells to dilute salt that has entered into root cells, and the resulting increased root pressure may push up dissolved salt from the roots to other organs to dilute the salt in the plant body [39]. Interestingly, despite the similarity in the major effect of both salt and water stress, i.e., cellular water deficit, differences exist in the response of particular genes to these stresses. For example, *AtPIP2;2* transcripts of *Arabidopsis* were noted to be up-regulated in response to salt stress [40] but downregulated in response to water stress in *A. thaliana* [40], while in the resurrection plant (*Craterostigma plantagineum*), *CpPIPa2* was up-regulated under dehydration but downregulated under salt stress [41]. Therefore, the results of gene regulation under these stresses must be considered separately.

An alternative method to identifying potential genes of interest is bioinformatics, i.e., the *in silico* analysis of genetic material and encoded protein products. Bioinformatics tools can include plant genome databases such as TIGR (http://www.tigr.org/tdb/e2k1/osa1/; http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=wheat; http://www.tigr.org/tigr-scripts/osa1_web/gbrowse/rice) and Gramene (http://www.gramene.org/Multi/blastview), as well as gene and protein analysis tools such as TMHMM Server v2.0 (http://www.cbs.dtu.dk/services/TMHMM/), ClustalW (http://www.ebi.ac.uk/clustalw/), WoLF Psort, (http://psort.nibb.ac.jp), TargetP 1.1 Server (www.cbs.dtu.dk/services/TargetP), SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP) and PredictNLS (http://cubic.bioc.columbia.edu/cgi/var/nair/resonline.pl). These tools allow researchers to extract, compare and compile information such as entire genome sequences, expressed nucleotide sequences, specific genes and their chromosomal locations, predictions of putative amino acid sequences and protein function, and finding homologous genes between different species, from vast amounts of genetic data. This approach can be used to link these types of findings to experimental data.

Regulation of MIPs During Salt Stress

The response of PIP expression to salty conditions has been investigated in a number of plants. Work on ice plant (*Mesembryanthemum crystallinum*), a salt tolerant plant due to its ability to accumulate salt throughout the plant and undertake Crassulacean Acid Metabolism (CAM), by western blot analyses of PIPs indicated that the protein MIPA was downregulated while MIPC was upregulated, suggesting different roles for these PIP members in response to salty conditions [47,48]. Other observations suggesting roles for PIPs in salt tolerance, summarised in Table 1, include upregulation of PIP1 during salt stress in rice [37], ice plant, tobacco [49] and *Arabidopsis thaliana* [40], or down-regulation in *A. thaliana* [40], rice [37], and the ice plant [48]. PIP2 transcripts have also been found to be up-regulated in *A. thaliana* [40, 45], rice [37] and radish [51], and downregulated in *A. thaliana* [40], rice [37], and the ice plant [37]. Tissue specific responses have been noted also, e.g., initial studies on a barley PIP gene *HvPIP2;1* identified it to be up regulated in shoots and downregulated in roots during salt stress [39]. It was, therefore, hypothesised that introduction of the antisense version of this gene in barley roots may improve the salt tolerance of the plant, and, indeed, over expression of this gene in transgenic rice plants reduced the salt-tolerance of the rice plant [64]. It has been proposed that a decrease in the expression of aquaporins after salt stress may prevent water loss from root tissue, but may be disadvantageous to growth under normal conditions

after extended periods [64]. Due to members of the PIP2 subfamily showing greater water permeability properties than the PIP1 subfamily, they probably possess different functions [35]. Various studies have confirmed this hypothesis; e.g., the application of NaCl resulted in an upregulation of RsPIP2-1 protein levels in comparison to no change in the levels of RsPIP1 in radish [51]. Another study suggested that *HvPIP2;1* from barley may have a role in response to abiotic stress due to its different regulation under salt stress in different tissues, whereas transcripts *HvPIP1;3* and *HvPIP1;5* appear to have roles unrelated to salt stress [39]. Fewer studies appear have been performed on TIPs in response to salt stress compared to PIP members (Table 1), but an upregulation of TIP transcripts in rice [43] and maize [53] has been noted. No downregulation of TIP mRNA has yet been observed, however, MIPF protein from the ice plant is downregulated during salt stress [47]. More research is thus required to determine which TIPs may have a role in salinity resistance. It should also be noted that some of the observations are inconclusive, with no clear patterns emerging in different species, and/or because transcripts are found to be regulated in different ways in different tissues; e.g., *HvPIP2;1* transcripts from barley are upregulated in shoots but downregulated in roots after salt stress [39]. Further, regulation can also differ between transcripts and proteins, e.g., MIPC (a PIP2) from the ice plant shows upregulation of transcripts but down-regulation of protein [47,48]; noting that in this case the experimental conditions were not exactly comparable for the two analyses. Studies using identical stress conditions covering all genes tend to illustrate patterns of significance; for example, the study of all thirteen PIP isoforms together from *A. thaliana* [40] was useful in identifying PIPs of interest (Table 1) for salt stress, and it would be interesting to extend this work to cover all thirty-five member family of MIP genes of this plant, as done for water stress studies [54]. However, the number of PIP and TIP transcripts noted in Table 1 that shows change in expression clearly indicates a role for these genes in salt tolerance.

Regulation of MIPs During Stress

PIPs have shown various responses to water stress and seem to play important roles in surviving these harsh conditions. One interesting group of plants for water stress study is the desiccation-tolerant group of species called the 'resurrection plants', their tolerance being partly attributed to their ability to form numerous small vacuoles in the bundle sheath cells [66]. It has been suggested that the high permeability of membranes to water may be critical to reduce the strain associated with rehydration.

As in case of salt stress, a number of specific MIPs have been shown to be regulated by water stress; for example, TIP3;1 has been localised to the above-mentioned small vacuoles in the resurrection grass *Eragrostis nindensis* and is hence thought to be important in this role [66], while upregulation of *Cp-PIPa* members 2, 6 and 7 in *Craterostigma plantagineum* [58] upon dehydration of excised leaves points to an important role for these PIP isoforms. Methods involving gene knockout have been particularly useful in identifying MIP roles in water stress; e.g., creation of a double antisense plant to 'knock out' the genes *AtPIP1;2* and *AtPIP2;3* is reported to have resulted in increased root growth and impaired recovery to water stress [33]. In a different approach, transgenic *A. thaliana* plants overexpressing *PIP1b* resulted in faster wilting and reduced growth performance during drought conditions [67]. One study in tobacco

plants investigating both aspects, i.e, introduction of antisense *BnPIP1,* which led to reduced tolerance to water stress, and overexpression of *BnPIP1,* which led to increased tolerance to water stress [56], points to an important role for this gene. Like the salt stress, the regulation of different PIP isoforms during water stress can vary; e.g., *AtPIP1;3* is downregulated while *AtPIP1;5* is upregulated during water stress (Table 2). The study concurrently comparing all *PIP* transcripts from *A. thaliana* (Table 2) illustrates that specific isoforms are upregulated or downregulated after the application of mannitol, and thus important in response to water stress [40]. Another study covering all thirty-five MIP genes of *A. thaliana* showed similar results after creating water stress, except that *AtPIP1;3* was downregulated and *AtPIP2;6* showed no change [54], suggesting some genes might be regulated by both stresses, while others are more specific to the nature of the stress. Other studies showing down- or upregulation of specific isoforms of TIPs after application of water stress in *A. thaliana* [54], rice [43], or sunflower (*Helianthus* sp.) are summarised in Table 2 [63,64]. However, it should be noted that it is sometimes difficult to directly compare the results of some of the experiments, due to differences in methodology of inducing water stress (e.g., application of mannitol, withholding water, or root exposure to air) or the tissues and species being studied [40,54,64]. Different methods may provoke different responses in gene regulation, as suggested by the upregulation of *AtPIP1;3* during exposure to mannitol [40], and its downregulation during discontinued watering [54]. More uniform methodology and functional studies are thus required to further investigate the roles of MIPs in response to water stress. Further, bioinformatics tools mentioned earlier can also be useful in stress-responsive gene identification. We used data from the sequenced rice genome (*Oryza sativa* spp *japonica* cv Nipponbare) using the TIGR Rice Genome Annotation Database to identify all rice MIP genes and to predict their gene and encoded protein features such as transmembrane helices and NPA motifs, substrate specificity, signalling and expression, the compilation resulting in the identification of altogether thirty eight MIP genes in rice, comprising of all four subfamilies. This, together with reports on experimental analysis of MIP gene functions, especially involving abiotic stress response, led to the identification of eleven rice MIPs of interest, i.e., showing differential expression under stress. Of these, only two (RWC1 and RWC3) had been tested for and shown to have water permeability, i.e., could be described as functionally active aquaporins [50,52]. Sequence alignments of their putative protein products against the rice MIPs identified in our study demonstrated high identities with some of the rice loci, and suggested that the rice OsPIP1;1 and OsPIP1;3 are aquaporins and may contribute to drought response. As our interests lie in wheat (*Triticum aestivum*), we are currently utilising this information in identifying homologous genes in wheat for future abiotic stress response studies.

Regulation of NIPs and SIPs During Water Stress

In contrast to PIPs and TIPs, only a few studies have investigated the roles of NIPs and SIPs. One such study demonstrated that *AtSIP1;1* was not regulated by water stress [54]. In another study, although *rMIP1* (a NIP) from rice was not affected by either treatment [43], *At-NLM1* (a NIP also) from *A. thaliana* was upregulated during water stress and downregulated during salt stress [42]. These results indicate that the NIP subfamily may be worth further investigation in studies of such stress response.

Additional Roles of Plant Aquaporins in Regulating Plant Water Relations

Water flows through living tissues in plants as cells lose water due to transpiration and the expansion of growing cells. Water uptake and its movement throughout the plant is required for a large number of cell turgor processes in plants including cell enlargement [68], stomatal movement [63], phloem loading [69] photosynthesis and transpiration [67], suggesting that aquaporins are vital for the survival of plants for such processes to continue. Indeed, a number of reports, summarised below, strongly suggest the importance of water transport via aquaporins in a number of diverse processes related to plant-water relations: (i) involvement of AthH2 (a PIP) from *A. thaliana* in cell differentiation [70]; (ii) possible requirement of Bn*PIP1* transcripts, induced by imbibition in Canola (*Brassica napus L.*) seeds, for water transport for the enzymatic metabolism of storage nutrients at early stages of seed germination [71]; (iii) suggestion that pre-harvest sprouting in wheat, a significant problem facing farmers and involving early seed germination correlated with uptake of water into the embryo, can be prevented by selection of lines that do not express aquaporin genes rapidly when seeds are exposed to wet conditions [72]; (iv) involvement of NtAQP1 (a PIP1) in leaf unfolding, as its expression oscillates with diurnal and circadian cycles coinciding with leaf unfolding, protoplasts expressing NtAQP1 show greater water permeability during leaf unfolding, and epinastic leaf movement is reduced in transgenic tobacco expressing antisense *NtAQP1* [73]; (v) role of aquaporins in stomatal movement, as transcript levels of *SunTIP7* appear correlated with fluctuations in stomatal conductance [63] in sunflower (*Helianthus annuus*); and (vi) occurrence of different TIP isoforms in different vacuoles in the same cell, thus acting as their protein markers and suggesting functional differentiation, e.g., α-TIP in the membrane of vacuoles that contain seed storage type proteins [74] and of autolysosomes [75], γ-TIP in lytic or degradative vacuoles [74,76,77] and δ-TIP in the membrane of vegetative storage protein vacuoles [74,78]. Expression of specific aquaporins in response to parasite infections has also been noted, e.g., of *LeAqp2* (a PIP1) from tomato (*Lycopersicon esculentum*) after infection with *Cuscuta reflexa* [79], and *TobRB7* (a TIP) of tobacco after infestation with root knot nematodes [31, 80], although the nature of such relationship is yet uncertain.

Looking at the extensive list of plant functions discussed above that are regulated by aquaporins, it is not difficult to envisage that the roles of these genes may not be limited to only salt and water stress response, and may in fact extend to any aspect of plant life that is dependent on water uptake and availability. Further work such as detailed analysis of roles of individual isoforms, definitive functional studies on the genes of interest, as well as study of any interactions with other non-MIP genes involved in stress tolerance would be required to test this hypothesis. The physiological effects of changes in MIP transcript and/or protein level during response to salt and water stress also need to be understood further, so that appropriate genotypes could be identified and exploited for plant breeding purposes, to improve stress tolerance.

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8 Biotechnology in Plant Tolerance to Heat and Drought Stress

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Introduction

High temperature and drought are two major environmental factors that can severely limit plant growth and productivity. These two factors are becoming increasingly important due to predicted global warming and the decrease in fresh water availability. Temperature elevation and water shortages may impose great challenges for sustainable agriculture. In addition, many regions and countries throughout the world, especially developing countries, have already endured the burden of heat stress and a limited water supply for years. Hope lies in modern advances in improving heat and drought tolerance in order to maintain plant productivity in unfavorable environments to meet the increasing demand for a growing world population. Use of stress tolerant plant species and cultivars has successfully increased agricultural production in the past and will continue to be an important approach in the future.

In order to survive under adverse conditions, plants have evolved morphological, physiological, and biochemical mechanisms as advantageous adaptations. Adaptive mechanisms to environmental stresses may vary largely among different ecotypes/cultivars and species, depending on the environmental selective pressure they were exposed to over the course of evolution. Several approaches that take advantage of plant adaptive mechanisms and genetic variability may be used to improve plant tolerance to abiotic stresses, including traditional breeding and genetic modification using biotechnology. Traditional breeding methods have been used widely for years in the selection of germplasm with improved stress tolerance. Breeding for improving drought tolerance in maize (*Zea mays* L.) was initiated in 1975 in CIMMYT. The progress using traditional breeding in improving stress tolerance is limited by many factors, such as lack of genetic variability or tolerant germplasm, and the complexity of abiotic stresses [1]. Using biotechnology to improve plant stress tolerance was first reported in 1993 [2] and has offered researchers methods to more efficiently investigate stress tolerance

genes compared with traditional methods. Furthermore, biotechnology has allowed for transgenic methods that circumvent the limitations of traditional breeding.

Current biotechnological methods are dynamic and ever changing due to their relatively young age, especially within the field of bioengineering stress tolerance into plants. With new discoveries and improvements of existing methodologies being rapidly exposed, the field of bioengineering plants is moving into a practical realm. For instance, adding foreign genes to a plant's genome is no longer the only option. Now, due to entire genome sequencing and functional genomics, researchers are able to manipulate gene expression of existing genes that confer desirable traits. Expression of foreign genes in plants is still a useful method to determine gene function and can be used in conjunction with other analyses. Mutational analysis such as gene disruption by T-DNA insertional knock out mutation can allow researchers to determine the phenotypic consequences of the loss or gain of function of specific genes. Methods like promoter tagging are currently being used to identify gene regulatory sequences in order to increase or repress gene expression as well as induce expression in a stress-, tissue-, or developmental-specific manner. RNA interference or gene silencing can also be used as a repression system for functional determination of existing genes. Reverse genetic approaches have allowed the study of genes without knowledge of their end products. Genomic and proteomic studies such as microarrays are new methods that are quick ways to identify genes and proteins that are differentially expressed under different environmental conditions.

This chapter will focus on current genetic improvement and biotechnological advances in the development of heat and drought tolerant plants for important crop species and model research plants. First, it is important to review the basics of physiological effects of drought and heat stresses and plant adaptive mechanisms to cope with heat and drought stresses in order to understand what genes are of interest for biotechnological manipulation. Subsequently, different genomic tools and technologies that are used in stress tolerance improvement will be discussed. Drought and heat stress may occur simultaneously in many cases. For an orderly discussion, effects of heat and drought stress and mechanisms of plant tolerance to these stresses are elucidated separately in this chapter. In addition, many physiological and biochemical processes change during plant exposure to drought or heat stress. It is not the intention of this chapter to provide a comprehensive review of all effects of drought and heat stress on plant growth. Instead, we will review only major physiological and biochemical factors that are involved in drought and heat stress adaptation and are utilized in genetic improvement in heat and drought tolerance using biotechnology.

Effects of Heat and Drought

The effects of heat and drought stress may vary between plant species, the extent of stress acclimation, and their interaction with other environmental factors. The severity of the stress also greatly affects the resulting physiological state of the plant. The effects of different abiotic stresses and subsequent plant responses exhibit significant overlap, observed by the cross tolerance phenomena, in which one stress can induce tolerance to a different subsequent stress [3]. For an orderly discussion, the following is organized by individual stress.

Heat stress

Heat stress induces numerous complex cellular changes. Photosynthesis is one of the most sensitive processes to elevated temperatures. Temperature-induced decline in photosynthetic rate has been associated with decreased photochemical activity of PSII [4]. In both C_3 and C_4 plants, high temperatures also affect carbon reduction and fixation processes, including reducing the activated state of ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) by decreasing the activity of Rubisco activase [5]. The extent of loss of activase activity is largely temperature dependent. Under moderate heat stress or at temperatures just above the thermal optimum of net CO_2 assimilation (30-40°C), activase complexes dissociate. At higher levels of heat stress above 42°C, activase becomes highly denatured to the extent that it is most often un-reversible [6]. Photosynthesis is also inhibited by the loss of affinity of Rubisco for CO_2 and the simultaneous decrease in solubility of CO_2 under high temperatures, causing CO_2 to be a limiting factor; oxygenase activity is increased to stimulate respiration, a less energy efficient process than photosynthesis [7].

Excessive energy from reduced electron transport in photosynthesis during stress can lead to an accumulation of excitation energy that can be dissipated by reduction of molecular oxygen, causing the production of reactive oxygen species [8] such as hydrogen peroxide (H_2O_2), superoxide (O^{2-}), hydroxyl radicals (·OH), and singlet oxygen ($O^1{}_2$) [9]. Accumulation of cellular ROS can lead to membrane lipid peroxidation, which often leads to leaf senescence [10,11]. ROS can also cause de-esterification of lipids, protein denaturation and nucleic acid mutation. Lipid peroxidation occurs under stressed conditions because ROS scavenging systems can become overwhelmed, imbalanced, or non-functional [12]. In addition to peroxidation, membrane physical state is also highly affected by hyperthermic stress conditions. The fluidity of lipids increases with increasing temperatures and this property has a large effect on the extent of lipid-protein interactions and on membrane structure, which can be distorted to form local non-bilayer structures [13] and can increase membrane permeability, resulting in leakage of ions and other cellular compounds. Membrane fatty acid composition may also change during heat stress, due to both membrane damage and altered gene expression [14]. The extent of protein damage depends on the severity of the stress and can range from slight damage to complete denaturation, with concomitant protein loss and increased protease activity [11].

Drought stress

One of the major effects of drought stress is cellular dehydration. It causes a reduction in water content or water potential of the cell. Such changes can affect a myriad of physiological processes including cell growth, cell wall synthesis, plastid formation, nitrogen metabolism, CO_2 assimilation, hydraulic conductance, and proline and carbohydrate accumulation, in order of most to least sensitive [15]. The effects of drought stress on these processes are largely dependent on the severity of the stress.

On the cellular level, membrane composition and properties are highly sensitive to dehydration. In response to moderate drought, the lipid content of membranes has been shown to decline. This decline is strongly correlated with the inhibition of lipid biosynthetic pathways as well as stimulations of lipolytic and peroxidative activities. Gigon *et al.* [16] showed that in *Arabidopsis*, total leaf lipid content decreased over time and with increasing stress severity

levels. The alteration of lipid composition was not observed until severe drought stress occurred. The compositional changes included an increase in desaturated fatty acids and an altered balance between monogalactosyl-diacylglycerol (MGDG) and digalactosyl-diacylglycerol (DGDG). Drought stress reduced the ratio of MGDG to DGDG. This ratio is important in determining the structure of lipid bilayers, since MGDG tends to form hexagonal phase structures and DGDG forms lamellar phases. Thus, the alteration of this ratio causes reduced cellular membrane stability and inhibition of proper functioning of photosynthetic membranes [16]. Membrane polarity is also affected by drought stress, under which a decrease in polar lipids can be observed [17].

Photosynthetic rate typically declines under drought conditions, but may quickly recover upon rehydration as long as the photosynthetic apparatus is not permanently damaged [18]. While reduction in photosynthesis under drought stress may be due to both stomatal and non-stomatal (metabolic) limitations, stomatal limitation of CO_2 supply may be more important during the early phase of drought and non-stomatal impairment becomes more pronounced following prolonged or severe drought stress. Similar [19] to plants under heat stress, under severe water limitation net photosynthetic rate may decrease due to the decrease in the activity of Rubisco and the abundance of Rubisco small subunit (rbcS) transcripts. In tomato *(Solanum lycopersicum)*, the mechanism of reduced activity is thought to be due to the presence of intracellular inhibitors such as CA1P and 'daytime inhibitor' [20]. These inhibitors are thought to bind to Rubisco in unstressed conditions to prevent the destruction of inactive Rubisco by proteases. Simultaneously, the same experiment was done on wheat (*Triticum aestivum* L.), however, the results did not conclusively show that the inhibitors were decreasing the activity of Rubisco [20]. The reduced turgor pressure that often results from water limitation can cause changes in chloroplastic pH and ion concentrations due to the increased permeability of chloroplastic membranes. It is thought that these changes can contribute to Rubisco inactivation [15]. There are also still some questions about the effects of water stress on cellular RuBP. The mechanisms of both decreased Rubisco activity and RuBP regeneration under drought stress are not well understood [21-23]

Water deficit is often associated with heat stress and the effects of these stresses sometimes are indistinguishable. Similar to heat stress, drought stress also causes the formation of ROS, membrane alterations, reductions in net photosynthesis, photochemical efficiency and chlorophyll content [24]. Proteolytic activity also increases during both abiotic stresses [25,26], as evidenced by the accumulation of ammonium compounds often observed under salt or drought stress [27].

Plant Adaptive Mechanisms to Heat and Drought Stress

Plants have evolved numerous morphological and biochemical adaptive mechanisms to cope with the effects of adverse abiotic conditions, including heat and drought. Each of the aforementioned effects caused by heat and drought illicit plant responses.

Heat stress

Membrane compositional changes

Membrane lipid composition and saturation levels are altered in response to various abiotic

stresses by the regulation of biosynthetic pathways. The enzymes responsible for lipid saturation changes are called desaturases, which introduce double bonds into fatty acid hydrocarbon chains. The genes encoding these enzymes may also be temperature regulated. Plant acclimation or adaptation to heat stress is generally characterized by an increase in the saturation level of lipids, which corresponds to a decrease in the presence of double bonds within fatty acyl chains and a decrease in fluidity. For example, the levels of lipid saturation and the production of saturated lipids such as linoleic and palmitic acids typically increase, whereas decreases in linolenic acid may be detected in different plant species, such as found in creeping bentgrass (*Agrostis stolonifera*) exposed to heat stress [28]. The enzymes encoding desaturases may be down-regulated during heat stress, causing the increases in fatty acid saturation [29].

Changes in the composition of cellular membranes have been shown to play a role in cellular sensing and signaling of temperature stresses. In cyanobacteria, simulated cold stress using chemically modified hydrogenation resulted in the increased expression of the *desA* gene, which encodes an acyl-lipid desaturase. Since compositional changes in membrane structure resulted in altered gene expression, the plasma membrane has been described as a primary cellular thermometer involved in thermal perception signaling [30]. Further studies of this cyanobacterium have led to the conclusion that, conversely to cold treatment, thylakoid membranes act as a cellular sensor of heat shock. The alteration of lipid state in thylakoid membranes caused signal transduction pathways to induce transcription of heat shock genes, whereas plasma membrane saturation did not affect the induction of such genes [29]. Whether the saturation levels of plasma membranes or chloroplastic membranes plays a greater role in heat tolerance is still not well understood.

Heat shock protein expression

Heat shock proteins (HSPs) are the primary type of proteins induced by heat stress to protect cellular constituents from heat damage and assist in recovery. HSPs have also been shown to be induced during other abiotic stresses such as drought, salinity, and excess light. HSPs have been labelled molecular chaperones because of their roles in assisting and protecting numerous other proteins in several ways, including ensuring proper folding of nascent polypeptides during translation, denaturation required for transport across membranes, stabilization of damaged proteins, and proper subunit assembly [31]. HSPs are named and classified by their molecular weight in kilodaltons (kDa). The common large HSPs include Hsp60 (chaperonins), Hsp70, Hsp90, Hsp104/ClpB, Hsp110. The small heat shock proteins (sHSPs), 12–43 kDa, function to protect cellular proteins from aggregation caused by heat denaturation by an energy-independent process until thermal conditions allow renewed cell activity. When this occurs sHSPs work in conjunction with large HSPs such as Hsp70 to release and refold proteins in an ATP-dependent process [32]. sHSPs are the most abundant stress proteins in plants often accounting for 2% of the total protein content in heat-stressed plants [33]. This high level of expression of HSPs is regulated by heat stress transcription factors (Hsfs), which are inactive until stress signals such as damage and hormone accumulation activate and translocate them to the desired cellular compartment [31].

Drought stress

Morphological traits

Many plant species are known to survive conditions of water limitation by preventing exposure to the stress conditions from the outset. This type of mechanism is known as drought avoidance. Drought avoidance mechanisms allow a plant to postpone tissue dehydration when available moisture is low by increasing water uptake and reducing transpiration. Plant species that live in harsh environments where selective pressures are high have evolved several drought avoidance traits. Many plants species are able to increase water uptake efficiency by developing deep and extensive root systems under periods of drought. The degree of deep rooting can be a function of the plant's root penetration ability due to the mechanical impedance of various soil types and root morphological characteristics such as length and density [34]. The mechanisms behind which plant roots can elongate even under severe water deficits are not completely understood. Despite decreases in turgor pressure, longitudinal cell wall extension is often enhanced under water stressed conditions for drought avoidance. This is possible by the gene up-regulation of expansins and xyloglucan endotransglycosylases (XET) during drought as well as increased activity of these enzymes in root meristematic regions [35].

Other avoidance mechanisms include reduction in water loss through the alteration of leaf morphology such as leaf curling and folding to reduce leaf surface area, and the presence of morphological characters such as leaf hairs, low stomatal density, and thick waxy cuticles. Some researchers also classify other plant characteristics as avoidance mechanisms including trichomes and sunken stomata [25].

Stress signaling and stomatal regulation

Plant survival of stressful conditions such as drought is governed by the capacity for quick recognition of the stress and the rate of induction of protective mechanisms. The rapid closure of stomata is crucial for plant survival in drying environments. Stomatal closure is often described as the first line of defense since its response to water deficit is much quicker than other physiological changes. Stomatal closure reduces transpirational water loss and reduces water consumption. It is believed that when roots are exposed to drought stress a chemical signal is transported to shoots, inducing stomatal closure. The involvement of root-to-shoot signaling in regulating stomatal behavior has been found to play important roles in plant tolerance to drought stress [36,37]. Abscisic acid (ABA) is considered as the primary chemical signal translocated from roots to shoots causing stomatal closure in response to soil drying [38]. Increases in ABA concentrations in guard cells triggers a signal transduction cascade, including promoting the efflux of potassium ions from guard cells, which causes reduction in turgor pressure of guard cells and ultimately the closure of stomata [39]. ABA also mediates cytosolic Ca^{2+} levels and triggers Ca^{2+} mediated pathways by regulating movements through Ca^{2+} channels. Cytosolic Ca^{2+} then transmits the signal to protein sensors such as calmodulin, Ca^{2+}-dependent protein kinases (CDPK), and phosphatases, which play roles in ion channel regulation [40]. Calcium binding proteins such as calcineurin B-like (CBL) proteins are also important in calcium signaling pathways and are thought to contribute to early stress-related transcription factor regulation [41].

Hormonal signals are complex and the hormone concentration may act independently to confer the signal or it may act in conjunction with other hormones and/or with other signals such as ROS levels [42]. Sharp *et al.* [43] has shown that hormones working in conjunction with each other is exemplified by the indirect role of ABA in water stress signaling by inhibiting the synthesis of ethylene [42,43]. Other signals in addition to hormone levels are involved in relaying root-to-shoot signals. For instance, water stress-induced ABA accumulation was shown to trigger an increase in the production of ROS [44], which would lead to altered gene expression patterns. As shown by studies of highly drought tolerant resurrection plants, ABA concentrations are the most highly affected by drought stress and ABA-dependent and ABA-independent signaling pathways are used to confer drought tolerance [45]. Changes in ABA, cytokinins, and ethylene are also observed in heat stress, however, hormone signaling pathways caused by heat stress are less well understood; it is thought that ABA plays a role in the induction of certain heat shock proteins (HSPs) [46].

Membrane composition, permeability and signaling alteration

Like heat stress, drought stress causes an alteration of the degree of saturation of membrane lipids. There is still debate on whether the membrane compositional changes are due to plant defense or due to damage caused by the stress. However, drought stress reduces the amount of the unsaturated lipid linolenic acid (18:3) in many different plant species [14].

Generally, membranes are composed of phospholipid bilayers, which are readily permeable to gases such as O_2 and CO_2, but are not readily permeable to water and hydrophilic ionic solutes. Transmembrane proteins such as ATPases, channel proteins and co-transporters mediate the transport of water and ions across cell membranes. Major intrinsic proteins (MIP) are a family of transmembrane channel proteins that regulate membrane permeability to water and other compounds. Aquaporins are intrinsic membrane proteins that are important members of this family, which account for 5 to 10 percent of the total protein within tonoplasts (Tonoplast Intrinsic Proteins, TIP) and plasma membranes (plasma membrane intrinsic protein, PIP) [47]. MIP proteins are most often tissue specific and their expression can be induced by various stimuli including desiccation, as exemplified by the upregulation of the aquaporin RD28 (PIP2) in *Arabidopsis* [48]. MIP upregulation is somewhat counterintuitive; however, membrane proteins also regulate the influx of water. Other MIP proteins are downregulated during drought stress as shown by the reduction of MIP mRNA transcripts observed for the NgMIP2, NgMIP3 and NgMIP4 genes of *Nicotiana glauca* [49]. Therefore, the differential expression of aquaporins and other MIP proteins is considered a plant drought tolerance mechanism that helps to retain cellular water levels.

In addition to altered composition and permeability, recent studies have implicated membranes to be signaling indicators of drought stress. In addition to secondary messengers such as Ca^{2+} and cAMP, lipids such as phosphatidic acid (PA) have been recognized as signaling molecules. PA is formed by the cleavage by phospholipase D (PLDμ) of structural lipids, such as those in membranes, to form PA and free polar head groups. The presence of free PA is a rapid and transient signal that triggers protein kinases and other cellular response mechanisms. Flux through the PLD pathway triggers an ABA response and the production of PLD is induced by ABA [50].

Differential protein expression

Under drought stress conditions various genes are up or downregulated, resulting in increased or decreased protein expression levels. Late embryogenesis abundant proteins [20] are a group of proteins that accumulate in response to several abiotic stresses, including dehydration, which have been shown to protect cytoplasmic structures and prevent protein aggregation [51]. This group of proteins is ubiquitous, highly conserved among plant species and are expressed under specific developmental or environmental conditions. LEA proteins are divided into five groups according to their sequence homology and biochemical properties. Group 2 is comprised of the dehydrins, which have been well-studied [25,52]. Dehydrins have been shown to act like molecular chaperones and help macromolecules maintain their structural integrity due to the high number of polar residues located on the protein surface [45]. Dehydrins also may help protect cellular membranes, as revealed by the maize DHN1 protein that binds to lipid vesicles to produce conformational changes at the membrane-water interface [53], and inhibit lipid peroxidation [54]. Other protective proteins are those that help to maintain the functionality of chloroplast localized stress proteins or chloroplast drought-induced stress proteins (CDSP) [17]. For instance, Rey *et al.* [55] identified the potato (*Solanum tuberosum* L.) CDSP 32, which was thought to play a role in the preservation of the thiol:disulfide redox potential of chloroplastic proteins during water deficit. The function of this CDSP 32 protein was later conclusively determined to be a thioredoxin involved in the defense against oxidative damage [56].

In addition to protective proteins, in order to tolerate drought conditions plants must possess mechanisms to repair damage caused by dehydration as well as mechanisms to prevent damage. Proteolysis or protein denaturation is typically stimulated by drought stress. Repair proteins and proteinase inhibitors have been identified in numerous species, which repair damaged proteins or inhibit proteolysis. For example, in cowpea (*Vigna unguiculata*), drought stress induced the expression of a multicystatin, which is a protein inhibitor of cystein proteinases [57]. Similarly, enzymes involved in the biosynthetic pathways to create repair molecules are upregulated in response to drought. The polyamine biosynthetic pathway has received attention recently due to the role of polyamines (PA) in the drought response. Some common plant PAs are putrescine, spermidine and spermine. These compounds are thought to have roles in various protective tasks such as affecting the physical state of the tonoplast by non-covalently binding to the negatively charged groups of membrane phospholipids, assistance in maintaining native protein conformation, and reducing the negative affects of altered pH due to osmotic stress, such as reversing H+-ATPase and H+-PPase inactivity [58].

Osmolyte accumulation

The active accumulation of solutes within plant cells to help maintain cellular water levels by altering the osmotic potential is called osmotic adjustment. The solutes that accumulate are termed osmolytes, which include sugars, polyols, proline, ammonium compounds and sulfonium compounds. Drought stimulates the production of these compounds by altered gene regulation and expression of the genes directly encoding or indirectly encoding end products involved in their biosynthetic pathways [25]. The effectiveness of osmotic adjustment in increasing cellular water content is often disputed because osmolyte accumulation is not always correlated with

high tissue or cellular water content. Therefore, the term compatible solutes is often given to accumulated osmolytes to account for their neutrality and their additional roles in protecting enzymes, ROS scavenging [17] and membrane stabilization [16]. An example of compatible solute is proline, which has been shown to maintain NAD(P)+/NAD(P)H ratios at normal levels during water stress. This enhances the oxidative pentose phosphate pathway to supply precursors for nucleotide synthesis and secondary metabolite production [59]. Some other well-characterized osmolytes are glycine betaine, putrescine and sugars such as fructans, trehalose, mannitol, raffinose and sucrose.

Antioxidant Protection to Heat and Drought Stress

Free radical accumulation caused by stress is both detrimental and beneficial to plant survival due to the damage they cause at high levels and their role in stress signaling, respectively. ROS, particularly H_2O_2, are primarily produced due to the enhanced enzymatic activities of plasma-membrane-bound NADPH oxidases, cell-wall-bound peroxidases and amine oxidases in the apoplast during the stress response. They are involved in signaling various defense mechanisms such as stomatal closure and root elongation, often by interaction with Ca^{2+} channels and other signaling proteins such as MAPKs [60]. Once the stress signal is perceived it is necessary for plants to remove these harmful byproducts during recovery.

Plants contain a wide range of ROS scavenging systems to prevent damage caused by ROS hyperaccumulation. The accumulation of ROS caused by heat and drought stress are alleviated mainly by the induction of gene expression coding for antioxidant enzymes such as superoxide dismutases (SOD), catalases, glutathione-S-transferases [61], ascorbate peroxidases (APX), and glutathione peroxidases that break down and remove ROS [25,62]. In addition, many non-enzymatic gene products have been shown to be involved in ROS scavenging either directly by actively scavenging or indirectly by inducing gene expression of other antioxidants. For instance, calcium, ABA, ethylene, and salicylic acid were all shown to protect plants from heat and drought stress-induced oxidative damage [10], as well as nitric oxide [63]. Other non-enzymatic ROS scavenging metabolites are isoprene [19], α-tocopherol [64], ascorbate (AA), reduced glutathione (GSH), and pigments such as carotenoids and flavonols [44]. There are also various compounds that induce the expression of antioxidant enzymes such as proline, which accumulates under drought stress conditions [65].

Biotechnology in Stress Tolerance Improvement

As discussed above, plants develop various mechanisms to cope with heat and drought stress. Numerous physiological traits and genes identified in stress-tolerant plants, with great diversity in regulation and the end products they code for, could be potentially used for the improvement of heat and drought tolerance in plants. Many key genes and regulatory factors have been engineered to improve the plant response mechanisms discussed above. Some of the genes that will be discussed have been well-characterized and studied for several years whilst others are still in the nascent stages of research. Some of the genes have profound effects on plant tolerance making them more favorable for possible use in field conditions, whilst others have less promise of field application. Nevertheless, even the analysis of genes with little prospect for direct use in the applied plant sciences increase knowledge of the overall biochemical

processes involved in heat and drought tolerance for the beneficial exploitation of plant response mechanisms.

Heat Tolerance

Membrane stability and compositional changes

The increase in the saturation level of fatty acids or in the content of saturated fatty acids in plant cell membranes observed during heat stress may be utilized as a stress tolerance trait for heat tolerance improvement. However, limited research has been conducted to confirm the direct correlation between membrane lipid saturation and heat tolerance. In tobacco, Murakami *et al.* [66] showed that knock-out transgenic plants in which the gene encoding chloroplast omega-3 fatty acid desaturase was silenced had a lower level of trienoic acid and were more heat tolerant than control plants [66]. Heat shock proteins (HSPs) are also being studied to confer superior membrane stability. Hsp17 and other molecular chaperones such as α-crystallin and GroEL, may regulate membrane fluidity by stabilizing the liquid crystalline state and reducing fluidity [32].

Protein expression

The bulk of research employing biotechnological methods for improved plant heat stress tolerance has been on the exploration of differential protein expression. In particular, HSPs are currently being researched extensively due to their diverse roles in cellular protection from heat damage. Several transgenic studies of both large and small HSPs have illustrated enhanced heat tolerance due to overexpression of HSP genes. An example of a sHSP being utilized is *Hsp17.7*. An increase in the abundance of *Hsp17.7* mRNA and protein levels resulted in a significant increase in thermotolerance and UV-B resistance in transgenic rice [67] and the introduction of the carrot (*Daucus carota* L.) HSP17.7 (DcHSP17.7) conferred heat tolerance to potato (*Solanum tuberosum* L.), a cool-season crop [68]. The mitochondrial small heat-shock protein (MT-sHSP) from tobacco was introduced into tomato under the control of the CaMV35S promoter. Transgenic plants overexpressing the MT-sHSP gene exhibited higher thermotolerance whereas the antisense plants exhibited higher heat susceptibility and all plants exhibited normal growth [69]. In addition, there have been reports of HSPs conferring tolerance to various other abiotic stresses including drought. For instance, the overexpression of NtHSP70-1, a tobacco heat shock protein gene, in the sense direction in tobacco conferred enhanced drought tolerance relative to wild type plants [70].

HSPs are upregulated or turned on during stress. In order for HSPs and other heat induced proteins to be created under heat stress, the thermally sensitive process of protein synthesis needs to be protected. Proteomic studies of heat tolerance maize have revealed that EF-Tu, the chloroplast protein synthesis elongation factor, accumulates under heat stress conditions. In addition to its role in polypeptide elongation, it is thought to accumulate because it acts as a molecular chaperone by complexing with and refolding denatured proteins [71]. Heterologous expression of the maize EF-Tu in *E. coli* enhanced the viability of the bacteria under elevated ambient temperatures [72] and mutational analysis of maize with reduced EF-Tu activity were less heat tolerant than wild type plants [73]. Also, *in vitro* chaperone assays of pre-EF-Tu indicated that the precursor did exhibit chaperone activity.

This assay also revealed that pre-EF-Tu did not require other chaperones or ATP in its protective role, which sets it apart from HSP chaperone activity [74]. This energy efficiency of EF-Tu relative to HSPs illustrates the potential of EF-Tu expression as a heat tolerance mechanism that could be utilized in transgenics.

Overall, HSPs have great potential as a stress tolerance trait for genetic modification because they are located ubiquitously throughout plant cells, including the nucleus, cytosol, mitochondria, and plastids, and because of their diverse roles in alleviating multiple stresses.

Drought Tolerance

Morphological characteristics

Genes controlling morphological traits associated with drought avoidance as discussed in the previous section may be used in genetic engineering to improve protection from dehydration damage. Zhang *et al.* [75] showed that in alfalfa (*Medicago sativa*) the constitutive overexpression of a transcription factor gene involved in the regulation of cuticular wax formation, WXP1, enhanced drought tolerance by delaying wilting and transgenic plants also showed quicker recovery after re-watering. Total wax accumulation was increased by approximately 30 to 40 percent without producing deleterious effects on plant health. Previous studies have identified numerous other wax-related genes, however, overexpression of most failed to increase wax accumulation. This study demonstrated that the degree of cuticular wax formation can be successfully increased by transcription factor activation of wax biosynthesis genes to enhance drought tolerance in important crop species like alfalfa.

Other regulatory elements such as different promoter and enhancer regions are also being exploited to increase plant survival of drought stress. Promoter trapping is currently being used as an effective way to identify genes responsive to specific stimuli. Cryptic promoters have been characterized using this method, which are 'pseudo-promoters' capable of driving the expression of otherwise promoter-less genes that are termed cryptic due to their often intergenic location [76]. Sivanandan *et al.* [77] have cloned and identified a novel root specific cryptic promoter from an intergenic region that is not associated with any specific gene. Root specific promoters have implications for enhanced drought tolerance by driving the expression of root growth genes such as hormones that would otherwise be detrimental if constitutive expression occurred.

Genes coding for expansins and xyloglucan endotransglycosylases (XET), which have been shown to be principal proteins involved in root elongation, may require the use of root specific promoters. Rochange *et al.* [78] observed stunted growth with shorter leaves and internodes in transgenic tomato plants (*Lycopersicon esculentum*) constitutively expressing an expansin gene, *CsExp1*, under the control of the CaMV 35S promoter. To circumvent this problem, Lee *et al.* [79] identified the first root-specific expansin gene, GmEXP1, in soybean (*Glycine max*). They found that expression levels of *GmEXP1* were very high in the roots of young seedlings, in which rapid root elongation was observed. In addition, *GmEXP1* mRNA was most abundant in the root tip meristematic regions, where cell elongation occurs. They also found that *GmEXP1* accelerated the root growth of transgenic tobacco (*Nicotiana tabacum*), implicating the great potential for use of this gene and similar genes in enhancing drought tolerance. Most recently, other proteins such as copper-containing amine oxidases (CuAOs)

involved in putrescine catabolism have also been shown to exhibit root specific increases in cell expansion in rapidly dividing areas [80].

Stomatal regulation

The turgor state of guard cells determines the status of stomatal apertures. Turgor pressure is regulated by the mechanisms described previously. Several genes involved in the cascading pathways leading to guard cell turgor fluxes have been identified and are being investigated for properties that cause stomatal closure when induced by water stress. For instance, *AtMYB61* is an *A. thaliana* gene that is the first R2R3-MYB transcription factor found to control stomatal aperture. This was discovered by reporter tagging and loss- or gain-of-function mutational analyses. Specific expression of the gene in guard cells resulted in altered relative amounts of open or closed stomata, respectively. Since AtMYB61 is normally downregulated by water stress in wild type plants [81], gain-of-function mutants that constitutively overexpressed the AtMYB61 gene had more closed stomata than wild type plants [82]. In addition to transcription factor regulation of stomatal pores, K^+ channels can be genetically manipulated for improved drought tolerance. Hosy *et al.* [83] and Becker *et al.* [84] have targeted and characterized a guard cell K^+ channel controlled by a gene named GORK that is regulated by ABA. In contrast to expression in other tissues, GORK expression as well as K^+ efflux activity through the GORK channel in guard cells is ABA insensitive, allowing the plant to adjust stomatal movement and water status control separately [84]. Ion channels regulating guard cell turgor such as this one could have great potential in conferring drought tolerance through the use of biotechnological methods.

Membrane stability, composition, and permeability

Enzymes involved in compatible solute accumulation and lipid biosynthesis and modification have been shown to play roles in stabilizing membranes and altering their composition during stress responses. Certain compatible solutes such as raffinose oligosaccharides and fructans have been shown to enhance membrane stability during stress. Fructans accumulate and are able to stabilize membranes by inserting between the polar head groups of phospholipids to help decrease electrolyte leakage and maintain bilayer structure [85]. Few plants accumulate fructans including members of Asterales, Liliales and Poales, therefore, several different studies have been conducted on generating transgenic plants that are able to produce fructans [86,87]. In these studies, emphasis has been placed on the ability of fructosyltransferase and ketose exohydrolase genes imparting cold tolerance, thus more research is needed on the ability of fructans to confer drought tolerance.

As discussed previously, the composition of plant membranes are altered by drought stress to reduce the level of unsaturated lipids. In tobacco, drought and osmotic stress tolerance were enhanced by the ectopic overexpression of genes coding for the desaturases FAD3 and FAD8 [14], demonstrating that genetically manipulating membrane composition by increased levels of desaturated membrane lipids could be an effective mechanism for drought tolerance.

Membrane permeability can be improved by the introduction of genes coding for aquaporins. These membrane proteins facilitate water movement through the polar regions of lipid membranes and could enhance drought tolerance by both increasing cellular water influx and

decreasing efflux. An example of an aquaporin that increases water influx is the plasma membrane aquaporin from *Brassica napus* (BnPIP1). The expression of sense and antisense cDNA transcripts of BnPIP1 in tobacco (*Nicotiana tabacum*) allowed Yu *et al.* [88] to determine the effects of this aquaporin on drought tolerance. Sense expression facilitated water transport through plasma membranes and enhanced drought tolerance whereas plants expressing antisense BnPIP1 showed morphological deformation, developmental delay and decreased tolerance to water stress [88]. Similarly, a rice (*O. sativa*) PIP aquaporin, RWC3, identified in a drought-tolerant cultivar has been shown to be induced by osmotic stress. When this gene was introduced into a drought-sensitive cultivar in conjunction with a stress inducible promoter, growth performance of this cultivar was improved during water stress [89]. However despite these successes, there are several intrinsic difficulties with manipulating aquaporin content, especially since aquaporins are two-way systems that could decrease cellular water content and have a reverse effect on drought tolerance. A study by Katsuhara *et al.* [90] showed that heterologous overexpression of a barley PIP homologue in transgenic rice decreased drought tolerance and raised the salt sensitivity of transgenic plants. Conflicting studies such as these show that isoform-specific functions exist and therefore, it is necessary to identify those that may enhance drought tolerance [91].

Protein expression

As discussed above, LEA protein expression is being widely studied due to its potential in conferring drought tolerance. In two separate studies, Park *et al.* [92] successfully transformed lettuce (*Lactuca sativa*) and Chinese cabbage (*Brassica campestris)* with a cloned group 3 LEA protein gene, ME-leaN4, from rape [88]. Transgenic lettuce and cabbage plants exhibited enhanced growth and delayed wilting under water stress conditions compared to wild type plants. Other studies of LEA proteins, including the dehydrin gene OsDhn1 expression in rice (*Oryza sativa*) [93] and CaLEA6 from *Capsicum anuum* transformed into tobacco [94], have had similar results but questions remain on whether or not the use of transgenics to express LEA proteins in various crops would be effective in enhancing drought tolerance in field conditions. Bahieldin *et al.* [95] have tested transgenic wheat plants expressing a HVA1 gene coding for a group 3 LEA protein in field conditions. They quantified characteristics such as total biomass, plant height, and grain yield, which were all greater in transgenic lines exposed to drought compared to wild type and control plants. Thus, LEA proteins seem to be a promising tool for the generation of drought tolerance species.

Other proteins involved in signaling and biosynthetic pathways are also being examined for use in enhanced drought tolerance. Mitogen-activated protein kinases (MAPKs) are involved in plant signaling in response to drought stress. Several MAPK genes have been cloned and have been shown to be activated by numerous stresses in addition to drought including pathogen invasion, salinity, cold and wounding [96]. Overexpression and repression analyses of a MAPK gene OsMAPK5 from rice showed enhanced and decreased tolerance to drought, respectively [97]. Mutation of CBL1, a calcineurin B-like (CBL) protein calcium sensor involved in calcium signaling during stress, impairs plant responses to drought. Conversely, overexpression of CBL1 reduces transpirational water loss and induces the expression of early stress-responsive transcription factors and stress adaptation genes in non-stressed plants [41,98].

The genes involved in polyamine (PA) biosynthesis are also targets of much research due to the role of PA in drought tolerance. Transgenic rice plants have been generated expressing the arginine decarboxylase gene isolated from *Datura stramonium* [99]. In this study, the levels of PAs and the enzymes involved in the PA biosynthetic pathway were monitored. The results showed that transgenic rice plants had higher levels of putrescine, spermidine and spermine and therefore, were more protected from drought stress than control plants.

Osmotic regulation

Plants naturally differ in their capacity to produce compatible solutes in terms of the amount and in terms of the type of solutes that they produce. Transgenics allows the assessment of which solutes are effective and at what expression levels. Glycine betaine (GB), proline, raffinose family oligosaccharides, fructan, mannitols and trehalose are several examples of osmolytes that are being introduced into different species.

Quan *et al.* [100] successfully engineered maize (*Zea mays*) to enhance glycine betaine synthesis by the introduction of the *bet*A gene from *Escherichia coli*. Within the glycine betaine synthesis pathway in *E. coli*, choline dehydrogenase, encoded by the *bet*A gene, oxidizes choline into betaine aldehyde. Choline dehydrogenase also catalyzes the oxidation of betaine aldehyde into GB. The presence of the *bet*A gene in maize enhanced GB accumulation and therefore, transgenic plants exhibited greater drought tolerance and had higher grain yield under drought stress than wild-type plants. In these plants GB was thought to protect the integrity of cellular membranes allowing for greater enzymatic activity [100]. GB accumulation has also been shown to enhance heat tolerance, as demonstrated by transformations of *Arabidopsis* plants with a gene for choline oxidase to produce plants that were more tolerant of high temperatures [101].

Proline accumulation has also been shown to effectively confer drought tolerance in several transgenic lines of different species. The D1-pyrroline-5-carboxylate synthetase genes, AtP5CS from *A. thaliana* and OsP5CS from *O. sativa*, were both effective in improving drought tolerance in petunia (*Petunia hybrida*) [102]. Soybean (*Glycine max*) plants were transformed with the cDNA for L-1-pyrroline-5-carboxylate reductase (P5CR), an enzyme involved in proline biosynthesis, in the sense and antisense directions. Sense transformants exposed to both heat and drought exhibited the least water loss, greatest proline levels, and had higher levels of NADP+ to act as electron acceptors for PSII and enhanced photosynthesis compared to the antisense plants [103,104].

Overexpression of AtGolS2, a stress-responsive galactinol synthase gene, in *Arabidopsis* led to increased levels of galactinol and raffinose, which conferred reduced transpiration and improved drought tolerance. Galactinol and raffinose were only detected in stressed plants, suggesting that they are specifically induced by drought stress [105]. Similarly, the expression of an *E. coli* trehalose-6-phosphate synthase and phosphatase genes in rice resulted in increased drought tolerance without stunting plant growth. Trehalose is a compatible solute known to have high water retention activity for osmotic adjustment and helps maintain the fluidity of membranes during water limitation [106]. The success of transgenically induced OA in reducing water loss under drought stress together with the low occurrence of abnormal growth caused by these molecules makes OA a prospective mechanism for enhancing drought tolerance in various plant species.

Multiple Stress Tolerance

As discussed earlier, there are often interactive effects of drought and heat stress on plant growth in natural environments. The effects on gene expression of heat and drought stress in combination may be different from each stress applied individually. Therefore, generalized response mechanisms common to different abiotic stresses should be used to effectively improve plant tolerance to multiple stresses.

Antioxidants

As discussed above, the removal of ROS is necessary to prevent oxidative damage caused by their accumulation. Plants do this by the production of ROS scavenging enzymes and metabolites. The combined effects of heat and drought, which are conditions that often occur together in nature, on gene expression have been studied by cDNA array methods. Rizhsky *et al.* [107] showed that some transcripts such as catalases, glycolate oxidases and thioredoxine peroxidase were induced by heat and drought independently, but suppressed by the combination of heat and drought. Antioxidant levels can be increased by the genetic manipulation of their precursors. Kocsy *et al.* [65] examined the effects of proline levels on the levels of the antioxidant homoglutathione (GSH) during simultaneous heat and drought stress, since both compounds have glutamate as a precursor. Transgenic plants containing the sense cDNA coding for P5CR had the lowest H_2O_2 content and the lowest injury percentage due to increased Pro and ascorbate (AA). The limited availability of glutamate as the result of increased Pro synthesis reduced the rate of GSH synthesis and content in the sense plants [65]. Thus, this study demonstrates that producing transgenics for one trait like proline synthesis may have negative effects on the production of other beneficial compounds. In this case, the increased amount of AA produced was able to compensate for the reduced amount of GSH for oxidative protection.

Among antioxidant mechanisms, H_2O_2 detoxification by different ascorbate peroxidases (APX) isoforms plays an important role in both heat and drought tolerance. Water deficit induced increases in transcript accumulation of APX genes in cowpea (*Vigna unguiculata*) cultivars were positively correlated to drought tolerance. Chloroplastic APX genes responded early to progressive water deficit, suggesting that the enzymes detoxify ROS at their production site [108]. CAT also removes H_2O_2 and the expression of CAT genes in wheat was found to be complexly regulated by both drought stress and circadian rhythms. H_2O_2 levels were not effectively reduced even though CAT activities doubled. Also, drought decreased the abundance of CAT transcripts in wheat [109]. Studies such as this one demonstrate the complexity of manipulating gene regulation and often expected results may not be observed. SODs also are highly upregulated during drought stress and have been shown to successfully reduce oxidative damage. Under the control of an oxidative stress-inducible promoter, rice plants expressing a pea manganese superoxide dismutase (MnSOD) in chloroplasts were shown to have less electrolyte leakage and higher photosynthesis rates than wild type plants [110].

In addition to important antioxidant enzymes like SODs and APXs, heat shock proteins and other cellular metabolites also play a role in ROS scavenging. For example, the small heat shock protein 21 (HSP21) plays a role in the protection of photosystem II from oxidative damage [111]. Nodulin proteins, which have been isolated from bean symbiotic root nodules (*Phaseolus vulgaris* L.) are similar to small heat shock proteins due to their domain structure.

Strong antioxidant defenses are required in root nodules because of the high respiration rates required for N fixation and the presence of heme molecules, which can convert H_2O_2 to hydroxyl radicals. The gene for nodulin 22 was overexpressed in *E. coli* and protection from oxidative stress was observed [112].

Hormonal signaling and sensitivity

Hormone changes are caused by environmental stresses and cause a complex cascade leading to plant defensive responses. Many stresses elicit an increase in ABA, which is required for survival of both heat and drought. Plant sensitivity to ABA can be manipulated for enhanced stress tolerance. For instance, era1 is an *Arabidopsis* gene that encodes the beta-subunit of a farnesyltransferase (AtFTB), which adds farnesyl groups to proteins. era1 mutants showed reduced wilting during drought stress, demonstrating that a negative regulator of ABA sensitivity must be farnesylated to modulate ABA response in *Arabidopsis*. Through transgenic insertion of the antisense form of the era1 gene coding for the farneslytransferase, it was shown that transgenic canola plants were more sensitive to ABA under drought conditions since reduced stomatal conductance and transpiration were observed [113]. Furthermore, this type of downregulation was conditional and reversible, illustrating the great potential of hormone regulatory system alterations for use as stress tolerance mechanisms [113].

From various promoter analyses, several ABA-responsive elements (ABREs) and ABRE binding factors (ABF) have been identified to be leucine zipper proteins that mediate stress responsive ABA signaling. The constitutive overexpression of ABF3 or ABF4 has shown that ABFs mediate stress-responsive ABA signaling in *Arabidopsis*. This was observed as ABA hypersensitivity, reduced transpiration, and enhanced drought tolerance of the transgenic plants [114]. Thus, the regulation and sensitivity of plants to ABA could be utilized for enhanced stress tolerance.

Transcription factors

Transcription factors are the stress response elements that perhaps have the most potential for enhancing tolerance mechanisms for multiple stresses. In *Arabidopsis*, transcription factor families ERF/AP2, bZIP/HD-ZIP, Myb, WRKY, and several classes of zinc- finger proteins, each containing a distinct type of DNA binding domain, have all been characterized. These transcription factors bind the stress-responsive *cis*-elements and activate the expression of target genes [96]. The target genes have end-products for various key players in the physiological response such as ABA.

In addition to the drought tolerance exhibited by the ABFs discussed above, Kim *et al.* [115] observed multiple stress tolerance in transgenic plants overexpressing ABF3. Transgenic lines were tolerant of low-temperatures, heat and oxidative damage. Similarly, overexpression of the pepper transcription factor CaPF1 in transgenic Virginia pine (*Pinus virginiana* Mill.) conferred multiple stress tolerance by increasing plant oxidative stress defenses [116]. Other *cis*-acting elements that have been the topic of much research are dehydration responsive elements and ABREs since many stress-inducible genes contain these elements in their promoter regions. Amongst others, NAC transcription factors, bZIP proteins such as TRAB1 and MYB activators bind to these regions and have been shown to upregulate certain stress responsive

genes to enhance plant defenses [117,118]. For example, Suzuki *et al.* [119] have reported that constitutive expression of the stress-response transcriptional coactivator multiprotein bridging factor 1c (MBF1c) in *Arabidopsis* enhanced the tolerance of transgenic plants to heat or osmotic stress alone, as well as the combination of both stresses. Most importantly, the expression of MBF1c augmented the accumulation of a number of defense transcripts in response to heat stress via the ethylene-response signal transduction pathway [119]. This result demonstrates the effectiveness of transcription factors in conveying broad range, multidimensional plant tolerance mechanisms, and it is commonly known that any type of broad resistance mechanism is more stable and effective than those conferred by single genes.

Conclusion

Mechanisms of plant tolerance to individual stresses, such as heat or drought stress have been well studied; however, they are far from being completely understood. Recent advances in stress physiology, molecular biology, and genetic modification using biotechnology have revealed important insights into plant adaptive mechanisms and have lead to significant improvements in stress tolerance of various plant species.

Huge advances in understanding transcription factor regulation are foreseeable in the near future since numerous heat shock genes and dehydration induced genes have been identified and shown to improve plant stress tolerance. Now, to move these technologies into the practical forefront controlled regulation and expression of these genes needs to be fully understood for efficient exploitation. Likewise, transgenic lines that have enhanced stress tolerance mechanisms tested *in vitro* need to be tested in the fields *in vivo*. The major obstruction to this move is public skepticism and environmental protection laws, which leads us to again conclude that controlled expression by the use of tissue specific integration to keep functional genes out of germ cells is of great necessity. Giant steps have been taken to make this a reality. For instance, site specific excision of extraneous recombinant DNA such as marker genes is now becoming possible due to new technologies such as the expression of site-specific recombinases, co-transformation of two T-DNA molecules, transposition-mediated excision, and site-specific recombination [120]. With political constraints tightening the budget for scientific research, the future must hold a shift into the practical realm for the discipline of bioengineering for plant stress tolerance to continue to grow and prosper.

In addition, most work on genetic improvement of plant tolerance has focused on single stresses or single genes. Future investigations should explore mechanisms of plant tolerance to multiple stresses since the interaction of different stress factors often occurs in natural environments. Stress tolerance traits are largely controlled by multiple genes and therefore, transformation of plants with multiple genes conferring stress tolerance may have great potential and may be a powerful tool for improving plant stress tolerance.

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9 Biotechnological Approaches to the Control of Plant Viruses

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Introduction

Plant viruses are among the most important of plant pathogens. The consequences of virus infestation on cultivated areas range from reduced crop quality to complete plant devastation. Virus specificity varies greatly, with some viruses able to colonize different hosts whereas others can only infect one defined species due to specific intricate interactions with the plant cell machinery. The virus/host interaction is dynamic with new virus varieties having different properties, for example exclusion from the original host plant. The appearance of pathogenic strains is especially important to agriculture. Disease management strategies need extensive knowledge of virus infection and its effect on host plants to allow the correct control procedures to be implemented. Reduction of crop loss is based on controlling pathogen dissemination rather than treatment of infected plants, as usually used with fungal or bacterial diseases. Different approaches have been used to diminish virus-spread throughout the plant and/or the plantation. Results from epidemiological studies might indicate the main route by which the virus reaches its host and the mechanism(s) of inoculation. Virus may be transmitted by contaminated seed, by vectors or during culture by normal agricultural practices. The use of certified seeds may significantly reduce the occurrence of certain viruses. Furthermore, vector population control and the implementation of 'clean' agricultural practices can considerably limit virus spread. In general, damage to the barrier composed of the cell wall and plasma membrane allows virus delivery into a viable plant cell, a process known as inoculation. Thereafter, should a compatible interaction occur between the virus and the plant cell, virus particles will replicate and spread within the host through plasmodesmata and vascular bundles. The intensity of these processes will depend on the relationship between the virus and the plant host. The intrinsic set of plant resistance responses aims to reduce virus replication. In some cases, breeding cultivars with elevated resistance levels represents a viable strategy to reduce

virus-induced crop loss. Another option is the use of attenuated virus strains to increase resistance responses which therefore act as vaccines. Advances in understanding the biochemistry of virus infection, such as RNA silencing, has resulted in potential new methods to efficiently limit viral diseases. In this review, a general discussion on plant responses to virus infection followed by outlining recent advances in natural and engineered resistance, the two major antiviral strategies used for crop protection. We have been studying the *Papaya meleira virus* (PMeV), the causal agent of papaya (*Carica papaya* L.) sticky disease. PMeV is designated to be a novel plant virus on account of its double-stranded RNA (dsRNA) genome and occurrence only at papaya laticifers. Some basic epidemiological information as well as the management of papaya sticky disease are briefly presented.

How Do Plants Depend Themselves against Viruses?

Viruses promote the infection of susceptible hosts by means of a variety of strategies. These strategies involve well-documented modifications in host plant cells that enhance infection. Initially, replication complexes produce abundant amounts of viral genome followed by the formation of new virus particles [1]. At this stage, some viruses are able to suppress plant gene silencing strategies [2-4]. Interference with cell cycle regulation [5] and cell-to-cell trafficking [6] as well as loss of photosynthetic activity [7] may also occur. Virus spread within the plant body exploits cell-to-cell and long-distance pathways. Plasmodesmatas are used to allow virus particles to move from the inoculation site to neighboring cells. Since plants control trafficking between cells mainly by alteration of the plasmodesmata diameter [8], some viruses synthesize specialized movement proteins that overcome this barrier and enhance the pore diameter [9]. Most viruses are loaded into phloem vessels in this manner, and transported with the photoassimilates to several plant organs [10]. At this stage many particles are available to be transmitted to another compatible plant, for example using an insect vector, thereby beginning a new life cycle. The host is not passive, however, during these processes as plants can fight infection if they possess resistance genes, the products of which are effective against invading viruses, or if general resistance mechanisms are activated [11]. Such responses may be general or specific responses and detailed knowledge of these are valuable in implementing the appropriate preventative measures.

The natural plant immune system is based on dominant and recessive resistance genes. In this model, plant dominant resistance genes (*R*) interact with pathogen avirulence (*Avr*) genes in an allele-specific genetic relationship. A form of localized programmed cell death, termed the 'hypersensitive response' (HR), is frequently observed in this type of interaction. Although it does not prevent host invasion by the pathogen, a basal response conferred by recessive resistance genes can also occur, thereby limiting the extent of invasion [12].

In general, all known dominant *R* genes have been grouped into eight classes based on their predicted protein structure. Only nine *R* genes have been isolated and sequenced: *N, Rx1, Rx2, Sw5, Tm2²*, *HRT, RTM1, RTM2* and *RCY1*. Most of the proteins for which these genes code possess putative N-terminal leucine-zipper (LZ) or other coiled-coil (CC) amino acids sequences, a centrally located nucleotide-binding site (NBS) sequence and C-terminal leucine-rich repeats (LRR) of various lengths [13,14]. To date, all *R* genes that confer resistance to viruses belong to the NBS-LRR class [14]. *R* genes that confer HR can recognize viral RNA

polymerase subunits, movement proteins, coat proteins (CP) and genomic segments as avirulence factors.

A mechanism to explain the genetics of Avr-R genes disease resistance would be that *R* gene products serve as direct receptors for pathogen-encoded *Avr* proteins [11,15]. An alternative mechanism would be that *R* proteins would form complexes that would recognize pathogen molecules in the initial invasion stages. Binding of pathogen molecules would lead to a sequence of cellular events that would constitute the defense response [16]. Evidence in support of such a mechanism is that HSP90 has been shown to be a critical component in immune responses triggered by the NBS-LRR proteins in plants [17,18]. It specifically interacts with RAR1 (required for MLA12 resistance 1), a member of the CHORD (cysteine- and histidine-rich domain) protein family [19]. Through its two zinc-coordinating domains, RAR1 can interact with Sgt1p, a component of the SCF (Skp1-Cullin-F-box) E3 ubiquitin complex [20]. Both RAR1 and SGT1 are required for signal transduction mediated by most *R* genes. Thus Hsp90 can contribute to the signaling pathways performed by other proteins related to *R* gene products [17]. Furthermore, it has been suggested that the effect of Hsp90 in disease response could be through direct or indirect modulation of NBS-LRR protein levels [17,21] and/or by suppressing viral resistance factors [21].

Less knowledge exists on plant responses controlled by recessive resistance genes. This resistance might be the result of a passive mechanism in which specific host factors required by the virus to complete its life-cycle are absent or present in a mutated form [22]. A translation initiation factor eIF4Ep from pepper [23], lettuce [24] and pea [25] impairs the potyvirus infection cycle via an unknown mechanism. Other proteins, e.g. OLE1 and TOM1, are involved in membrane structure and distribution of virus proteins targeted to vacuolar membranes respectively [26,27]. OLE1 is a Δ9 fatty acid desaturase that converts saturated to unsaturated fatty acids and is a major determinant of membrane fluidity. In the case of tobamoviruses, TOM1 was shown to interact with both TOM2A [28] and a polypeptide sharing helicase domain [29], consistent with the idea that membranes are of universal importance for positive-strand RNA replication of viruses [12].

Crop Protection Based on Natural Resistance

The use of virus-resistant cultivars is a cheap and effective approach to reduce the economic loss caused by plant viruses. In contrast, breeding for resistance is a long and costly process as the selected variants must have durable resistance to the target virus(es), at least throughout the life of the cultivar. Virus resistance can be either specific or non-specific. Specific resistance occurs when only one virus isolate from the group sampled from different hosts and geographical regions is able to infect the resistant cultivars. Non-specific resistance occurs when the resistance is effective against all the virus population. As a consequence of their error prone polymerases and the lack of a proof-reading mechanism during replication, viral pathogens have a high mutation rate [30]. The stability of host resistance depends on whether a new virus mutant emerges and overcomes the artificially selected or naturally achieved resistance [31]. The durability of host resistance will therefore be determined by both virus and host factors. In general, it will depend of the ability of the host to resist new virulent strains from the virus population.

Breeding resistant plant cultivars based on recessive genes may show more durable resistance than those based on dominant genes [32] since recessive resistance is due to loss of factor(s) essential for virus multiplication in the host cells [33]. The virus therefore needs to overcome the function of this missing factor to defeat host resistance [31]. Dominant resistance is generally less durable as virus mutations more easily suppress the interaction between plant resistance factors and virus avirulance factors [31], although in some cases the resistance remains useful for many years [34]. For example, the dominant *I* gene that protects *Phaseolus vulgaris* against BCMV and a number of other viruses has been used in Snap Bean breeding for decades [35]. Dominant resistance is preferred in breeding programs because it targets precise pairs of host genes [12] facilitating plant selection.

Crop Protection Based on Engineered Resistance

The majority of virus-resistant transgenic plants can be considered to be the result of pathogen-derived resistance (PDR) [36] brought about the expression of viral sequences in plant cells leading to plant protection. A pre-requisite for the use of PDR is that no interference with essential host functions should occur. PDR can be separated into protein-mediated resistance and nucleic acid-mediated resistance. Among the viral proteins used for PDR are replicases, movement proteins, proteases and most often, coat protein(s) (CP) [37]. The observation that transgenic RNA, rather than the expressed viral proteins was responsible for the observed resistance, created new opportunities based on RNA-mediated resistance [38,39]. An overview of the main mechanisms and applications related to these two types of engineered resistance are presented in the following sections.

Protein Mediated Resistance (PMR)

The initial report on PMR used *Tobacco mosaic virus* (TMV) CP gene expression to produce resistance in tobacco plants [40]. Since then, a number of studies have used PMR to confer plant resistance to a variety of viruses [41,37]. Viral coat protein-mediated resistance can provide either broad or narrow protection [37]. Thus the CP gene of *Potato mosaic virus* (PVY) strain N605 provides resistance in transgenic potato plants against this virus strain and also to the related strain 0803 [42]. Similarly, transgenic tobacco plants expressing a TMV CP gene are resistant to TMV and other closely related TMVs [43]. In contrast, the CP gene of *Papaya ringspot virus* (PRSV) strain HA provided resistance in papaya only against this HA strain [44], although it is unclear whether resistance was due to the CP itself or to the gene transcript [45]. CPs have roles additional to acting as CPs during the life cycle of a virus. Thus TMV CP was shown to enhance the production of movement proteins and coordinate the formation and size of virus replication complexes [36]. Transgenically expressed CP genes have been reported to interfere in this process leading to virus resistance as well as reducing the production of movement proteins thereby limiting the spread of cell-to-cell infection [47]. It has also been shown that the production of TMV CP in engineered plants interferes with TMV assembly [43]. This mechanism is able to confer resistance to a number of viruses including PVX, AlMV, CMV and TRV [48-50]. Using the TMV mutant (T42W) CP gene, alterations in virus disassembly were also seen.

Complete or partial viral replicase genes have been shown to confer immunity to infection. This is generally limited to the virus strain used to provide the replicase gene [45]. Thus mutant replicases from *Cucumber mosaic virus* (CMV) subgroup I conferred high levels of resistance in tobacco plants to all subgroup I CMV strains but not to subgroup II strains or other viruses [51]. Similarly, a mutant, but not a wild type replicase, conferred resistance to infection against PVY [52] and AIMV [53]. It has been proposed that this replicase-mediated resistance is brought about by repression of replication due to the transgene protein interfering with the virus replicase, possibly by binding to host factors or virus proteins that regulate replication and virus gene expression [45].

Viral movement proteins (MPs) allow infection to spread between adjacent cells (cell to cell) as well as systemically (long distance). Transgenic plants that contain mutant MPs from PMV show resistance to several TMVs as well as to AIMV, *Cauliflower mosaic virus* (CaMV) and other viruses [54]. Similar results were found for mutant MPs from *White clover mottle virus* (WClMV) [55], suggesting that such mutant proteins impair virus movement. The use of mutated MPs could therefore lead to transgenic plants that efficiently inhibit local and systemic spread of many different viruses.

The evaluation of mutant genes coding for CP and other viral genes used to confer PMR is of special interest for the commercial release of transgenic plants. It was shown that the molecular interaction between challenging viruses and transgenic plants can lead to heterologous encapsidation, complementation and recombination [56]. This has raised concerns on the potential biological and environmental risks associated with virus-resistant transgenic plants. Heterologous encapsidation occurs when closely related viruses use the functional viral CPs expressed in transgenic plant cells [57]. Transgenic CPs can transfer functions like vector [58] and host [59] specificity. Similarly, complementation occurs in transgenic plants if the transgenically expressed protein complements a mutant virus, which is defective in one or more genes. One method to prevent this phenomenon would be to abolish, by mutation of specific amino acids, the ability of transgenic CPs to form virus particles or the specific function of complemented proteins [56].

Nucleic Acid-Mediated Resistance (NAMR)

Pathogen-derived resistance has also been achieved through the expression of virus sequences, the acquisition of resistance being dependent on the transcribed RNA. This RNA-mediated virus resistance can be considered to be an example of post-transcriptional gene silencing (PTGS) in plants [60,61]. Napoli *et al.* [62] firstly reported PTGS in *Petunia hybrida* transgenically expressing the chalcone synthase gene. They observed a co-ordinated and reciprocal inactivation of the host gene and the transgene encoding the same RNA. This process has been called RNA silencing or RNA interference (RNAi) [63] and occurs in a variety of eukaryotic organisms [64,65]. The silencing process involves the cleavage of a dsRNA precursor into short (21-26 nt) RNAs by an enzyme, Dicer, that has RNase III domains. These RNAs are known as short interfering RNAs (siRNA) and microRNAs (miRNAs). Both siRNA and miRNA are able to guide an RNA-induced silencing complex (RISC) to destroy single-strand cognate RNA [66,67]. In addition, longer siRNAs (24-26 nt) have been shown to result in methylation of homologous DNA causing chromatin remodeling and transcriptional gene silencing (TGS).

In contrast, the closely related miRNAs cause the inhibition of translation by specific base paring with the target mRNA.

RNA silencing was first recognized as an antiviral mechanism that protected organisms against RNA viruses [68] or the random integration of transposable elements. However a general role for RNA silencing in the regulation of gene expression only became evident [67] after it had been demonstrated that specific short miRNAs precursor molecules (fold-back dsRNA) were actively involved in RNA silencing in plants and animals [69]. Several miRNA genes are evolutionarily conserved. Their function in plants is mainly to cleave sequence-complementary mRNA, whereas in animals such as *Caenohabditis elegans*, they appear to predominantly inhibit translation by targeting partially complementary sequences located within the 3′ untranslated region of mRNA [67].

Plant RNA silencing appears to be more diverse in comparison with other organisms. Some aspects of silencing are common for all eukaryotic organisms (e.g. the requirement of Dicer and Argonaute proteins). Sequence-specific DNA methylation (RNA-directed DNA methylation – RdDM) can be induced by dsRNA molecules in various plant systems and in response to various dsRNA inducers [70]. It has been suggested that RdDM also occurs in mammals [71] but not in fungi [72]. Silencing in plants is systemically transmissible within the plant body and can spread from the initial genomic target region to adjacent 5′ and 3′ non-target sequences [73,74]. A similar process appears to be absent in mammals and insects but occurs in *C. elegans* [66]. Furthermore, the size of siRNAs can vary from 21 to 25 nt in different species. In plants, siRNAs with 21-nt and 24-nt are found [75] but only two sizes, 21 nt and 25 nt, are present in the fungus *Mucor circinelloides* [76], whilst only a ~21 nt species of small RNAs appears to be present in animals [4]. In all organisms diverse proteins interact among themselves and with nucleic acids leading to different RNA silencing pathways.

Proteins Involved in Plant RNA Silencing

Several silencing-associated protein factors have been identified in plants. To date, Dicer-like (DCL) proteins, Argonaute (AGO) proteins and RNA-dependent RNA polymerases (RdRP) have been reported to play key roles in RNA silencing. However, RNA helicase and other proteins such as HEN1 and HYL1 are also involved [66]. RdRPs are particularly important in plant silencing in that they copy target RNA sequences to generate dsRNA [73] and that they are also required for chromatin modification [77].

Arabidopsis thaliana and rice encode for four DCL proteins with distinct functions. DCL1 together with HEN1 [78] and HYL1 is involved in miRNA biogenesis [79]. It appears to function in the nucleus, processing both miRNA primary transcripts and precursors [80]. Purified DCL1 from *A. thaliana* extracts was shown to be involved in the production of 21 nt siRNAs [81]. This enzyme is structurally and functionally similar to *Drosophila* Dicer-1 and human Dicer and is composed of two RNaseIII domains and a dsRNA-binding domain, a RNA helicase domain and a PAZ domain [82]. The PAZ domain, characteristic of enzymes that process small dsRNA, binds to the 2 nt 3′-overhang of dsRNA termini [83]. Its absence seems to be a typical aspect of long-dsRNA-processing enzymes [84]. DCL2 has been implicated in viral siRNA and the loss of function of this enzyme leads to reduced siRNA levels and increased virus susceptibility [85]. In *A. thaliana*, DCL3 is required for chromatin silencing through DNA

methylation, and is also required for the production of endogenous (transposons) siRNA [85]. DCL3 is the unique or the predominant enzyme that produces 24 nt siRNAs [81]. DCL4 is the only one that lacks a PAZ domain [83]. Recently, *A. thaliana* mutant in DCL4 was identified and analyzed [85]. This mutant lacks each of three families of 21-nt trans-acting siRNA (ta-siRNA) and possesses elevated levels of ta-siRNA target transcripts. Likely mi-RNA, ta-siRNAs acts to guide target mRNAs cleavage.

In animals, siRNAs generated by Dicer enzymes associate with RISCs, which recognize the target RNA. The enzymatic activity (Slicer) of the RISC is responsible for the cleavage of homologous viral RNA or mRNA. AGO proteins are the main candidate that perform this function. They possess two conserved domains: PAZ and PIWI [86]. The PIWI domain has been implicated in interacting with Dicer in complex formation. In mammals, it was shown that AGO2 contains the catalytic activity (Slicer) of the RISC and is directly responsible for mRNA cleavage [87]. Although the identity of plant Slicer is not known, at least ten AGO proteins are present in the *A. thaliana* genome [88]. AGO1 has been proposed as a Slicer candidate since accumulation of miRNAs is decreased in *ago1* mutants, this being accompanied by increased levels of mRNA from target genes [89]. Furthermore, it has recently been shown that AGO1, miRNAs and transacting siRNA may associate *in vivo*, with the complexes formed able to cleave the target mRNAs *in vitro* [81]. These results suggest that AGO1 is a key component of the *A. thaliana* RISC and is one of the *A. thaliana* Slicers [88]. When compared to human AGO2, eight *A. thaliana* AGOs (AGO1, AGO4, AGO5, AGO6, AGO7/ZIP, AGO8, AGO9 and ZLL/PNH) contain the DDH motif that characterizes the catalytic active site of Slicer. This suggests the existence of multiple other Slicers in *A. thaliana* besides AGO1 [90]. This large number of Slicers also suggests that different AGOs might regulate gene expression in specialized tissues or at particular developmental stages [88].

RNA Silencing Pathways

Three RNA silencing pathways have been described in plants [66]. These are cytoplasmic siRNA silencing, important in virus-infected cells, the silencing of endogenous mRNAs by miRNAs and a third pathway associated with DNA methylation and the suppression of transcription. These pathways all begin with the production of RNA transcripts of the organism genome with complementary or near-complementary 20 to 50 bp inverted repeats that can form dsRNA hairpins [67]. Such transcripts are considered miRNA precursors. Their maturation process involves Dicer-like proteins that possess dsRNA-specific RNase III-type endonuclease activity and dsRNA binding domains. Initial processing by Dicer occurs in the nucleus of the cell and the miRNA precursor is then exported to cytoplasm by means of nuclear export receptors such as the exportin 5 protein [91]. Once in the cytoplasm, the miRNA precursors is further processed by Dicer yielding miRNA duplexes of about 21 nt length [92]. Other sources can lead to the presence of dsRNA molecules in the cytoplasm. Thus they can be produced by RNA template derived RNA polymerization, e.g. from viruses, or by hybridization of overlapping transcripts from repetitive sequences such as transgene arrays or transposons [67]. Furthermore, they can be artificially introduced. Such dsRNAs lead to siRNAs production, which generally guide mRNA degradation and chromatin modification. As discussed above, four different Dicer genes are reported in plants, with each Dicer preferentially processing

dsRNA from a specific source. For example, DCL1 and DCL4 process miRNA precursors, whereas DCL2 and DCL3 are involved in the production of siRNAs from plant viruses and from repeated sequences respectively [67].

After the production of small RNAs (siRNA and miRNA), the RNA molecules are associated in ribonucleoprotein particles, which are subsequently rearranged into RISCs [97]. At least one member of the AGO protein family is present in the RISC, probably interacting directly with the target RNA in the complex. The AGO PAZ domain specifically recognizes the terminus of the base-paired helix of siRNA and miRNA duplexes [83] although the functional form of the RISC contains only single-stranded small RNAs. AGO proteins either bind preferentially to small RNAs of a specific sequence, or use specific adaptor proteins that were associated with dsRNA in its production site [67]. The described interaction with PAZ ensures the safe transitioning of small RNAs into the RISC by minimizing the possibility of unrelated RNA-processing or RNA turnover products entering the RNA silencing pathway. The small RNAs in the RISC guide a sequence specific degradation of complementary or near-complementary target mRNAs. Using a *Drosophila in vitro* system, it was shown that the target mRNA is cleaved in the middle of complementary region, ten nucleotides upstream of the nucleotide paired with the 5′ end of the guide siRNA [92].

The first evidence for miRNA-guided translational regulation was that miRNA targeted to a specific *C. elegans* gene reduced protein synthesis without affecting mRNA levels [69]. Similar processes also occur in plants. Although the mechanisms of translational repression are poorly understood, miRNAs appear to block translation elongation or termination rather than translational initiation [94].

RNAi can also induce gene repression at the transcriptional level through chromatin remodeling. Some regions of the chromosome structure are more loosely packaged (transcriptionally active euchromatin) whereas other regions are more tightly packaged (transcriptionally silent heterochromatin) [95]. Heterochromatin formation in plants and animals is associated with cytosine methylation [96] and this covalent DNA modification can be induced by plant or viral RNA. Thus RNA viruses have been shown to trigger methylation of identical DNA sequences present in the host genome [97,98]. Cytosine methylation in plants is brought about by CG methyltransferases [99] and cytosine methyltransferases [100]. A dense methylation pattern was observed in a RNA virion-infected tobacco system, with almost every available cytosine in the target transgene sequence methylated [101], suggesting that trigger RNAs efficiently recruit methyltransferases to establish and maintain methylation of target DNA sequences. Interestingly, in *A. thaliana* some cytosine methyltransferases are dependent on the H3 K9 methyltransferase KHP/SUVH4 [102,103] suggesting that histone methylation might be a prerequisite for DNA methylation. Alternatively, DNA methylation might trigger transcriptional silencing thereby causing enrichment of H3 K9 mRNA, which would then recruit other methyltransferases possibly to maintain the silent state [96].

Use of RNA Silencing to Biotechnological Control of Virus Disease

Enhanced resistance of transgenic plants to viruses has been brought about by expression of sequences able to trigger RNA silencing [104,105,106]. However, the possible environmental risks and the difficulties of transforming some species are obstacles to the application of this

technology. Strategies that confer RNA silencing such as dsRNA molecules of viral origin could prevent undesired consequences in hosts with unmodified genomes. Thus RNAi was synthesized in *C. elegans* incubated together with *E. coli* expressing a dsRNA corresponding to a specific gene [107]. An alternative method for the production of resistance in transgenic plants is the use of *Agrobacterium tumefaciens* to express dsRNA molecules [108]. Thus expression of a dsRNA coding for green fluorescent protein (GFP) in *N. benthamiana* tissues that also had the GFP gene present resulted in inhibition of GFP production. GFP synthesis was not inhibited when the *N. benthamiana* strains used either carried plasmids coding for GFP-specific dsRNA molecules or for viral suppressors of RNA silencing.

Strategies using exogenously supplied dsRNA have already been used to combat virus infestation in plants. *E. coli* was used to produce large amounts of dsRNA coding for partial sequences of two different viruses, *Pepper mild mottle virus* (PMMoV) and *Plum pox virus* (PPV) [109]. Simultaneous injection of dsRNA together with purified virus particles resulted in the inhibition of both viruses. Interestingly, resistance to infection was also observed when the crude bacterial preparations were sprayed onto the *N. benthamiana* leaves. These data suggest a simple, economic and effective application of RNA silencing technology. In the near future, we believe other such simple approaches to induce and enhance the efficiency of RNA silencing will emerge, leading to large scale applications of this sophisticated molecular pathway.

Risks Related to Genetically Engineered Plants

The main risks associated with genetically engineered plants [37] are the transgenic expression of viral genes in a compatible host, which can directly interfere with the life cycle of other viruses. A normal transgenic protein, for example those related to cell-to-cell and long-distance movement proteins, may complement defective viral proteins. Similarly, heterologous encapsidation using viral coat proteins expressed in the host represent a possible alteration in the process of transmission and host specificity that can contribute to infection. The natural process of gene flow between crop plants and their wild relatives can potentially alter the plant genome. Two possible problems are the fixation of crop genes in small populations of wild plants leading to a loss of biodiversity and consequent population extinction, and increased 'weediness' of wild relatives of the crop plant brought about by gene introgression resulting in plant growth in undesirable locations. This, however, would only occur if the transgene conferred an advantage that overcame a population size limiting factor, which would result in increased gene prevalence in the wild population. If the transgene were to confer resistance to conditions established by human activities, resultant problems could be controlled. If the transgene were to confer resistance to viruses, other pathogens or climatic conditions, the problems are far more complex as the selection pressure cannot be controlled.

Recombination, a covalent joining of nucleic acids that were not previously adjacent [37] might also allow the flow of plant genes to the virus genome. Recombination is seen to occur by a copy-choice mechanism during virus replication, involving one or more changes of template while the replicase complex synthesizes RNA complementary to the template molecule. Different types of recombination occur in the viral RNA genome, i.e. between identical sequences at equivalent sites (homologous recombination) or between unrelated sites that lack appreciable sequence identity (nonhomologous recombination). Reports have identified the

incorporation of chloroplast tRNA and cellular mRNA coding for an hsp70 homolog in the virus genome [110,111]. The advantages of recombination to the virus include elimination of deleterious alleles and creation of new variants. Indeed, sequence comparison has suggested that recombination might play a key role in viral evolution [112]. The susceptibility of virus resistant transgenic plants to recombination and the resultant emergence of new virus diseases is therefore of particular importance to the genetic engineer. It must be pointed out that recombination can also introduce point mutations and other errors into the viral genome, leading to a loss of viral fitness.

Papaya Meleira Virus (PMeV): A CASE STUDY OF A NOVEL dsRNA VIRUS AFFECTING PAPAYA

'Sticky disease' or 'meleira' is an important phytosanitary problem in papaya production in Brazil, mainly in Northern Espírito Santo and Southern Bahia States, the main producer regions. After initial reports of its occurrence in these two States [113,114], the disease was disseminated to other regions in Brazil where up to 100% of the plants in some orchards were affected [115]. Nowadays, sticky disease represents one of the main deleterious factors to papaya culture. Flowering of infected plants is followed by exudation of a fluid and watery latex from the fruits and leaves [116]. Exudation of latex occurs naturally in papaya after incision as all plant tissues possess laticifers, which form a complex array of cells able to produce latex. Normally, these cells are under high turgor pressure, and the latex produced has a milky consistency. Latex extrusion from healthy plants occurs normally through tissue lesions whereas that in sticky diseased plants occurs spontaneously [116]. Once exposed to the atmosphere, the latex is oxidized leading to a 'sticky' dark coloration on the fruit and small necrotic lesions in the young leave edges [116]. This last feature together with spots in the fruit pulp and ring, observed in a more advance stage of the disease, compromise the fruit commercially.

Different possible solutions were considered to elucidate the papaya sticky disease etiology [113,117]. After electron microscopy [118] detected the occurrence of approximately 50 nm diameter virus-like particles in diseased plant latex and laticifers, the isolation of approximately 12000 bp dsRNA molecules from infected tissues reinforced the possible viral etiology of the sticky disease. Unlike most plant RNA viruses, where dsRNA molecules are formed in specific steps during virus genome replication, virus particles from sticky diseased plants are present only with a complete genome [118,119]. After healthy papayas were found to become diseased after inoculation with purified virus particles, the virus was named *Papaya meleira virus* (PMeV) [119]. Recent electron microscopy and molecular data indicate that the viral particles are strongly linked to the polymers present in the latex (Figure 1), possibly as a protection mechanism or to aid viral transport [126]. Interestingly, the morphology of the polymers and the physiology of the laticifers were altered by the virus (Figure 1). Several other plant species were tested for their susceptibility to PMeV. Viral dsRNA could only be extracted from *Brachiaria decumbens* although these plants remained free of symptoms [119]. Its unusual genome and occurrence only in papaya laticifers make PMeV distinct from any other plant virus.

By mapping papaya sticky diseased plants in the field on a monthly basis, Ventura *et al.* [115] observed that disease dissemination occurred along planting lines, suggesting that PMeV propagation was induced by culture methods, although other epidemiological studies strongly

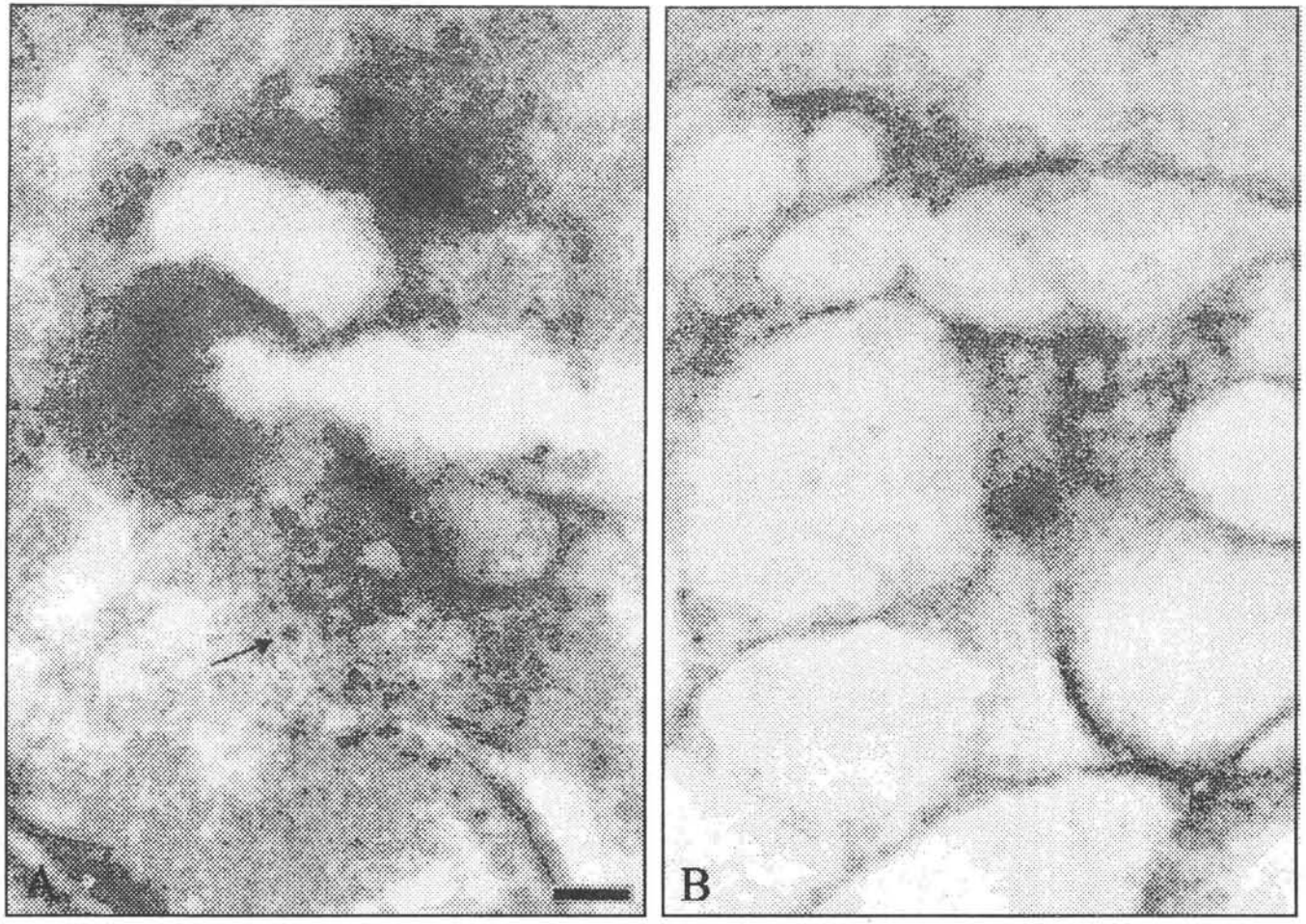

Figure 1. Transmission electron microscopy of C. papaya latex. (A) Latex from papaya sticky diseased fruits showing PMeV particles linked to the altered polymers surface (arrow) and control (B), latex from healthy papaya fruits; Barr = 200 nm.

indicated the involvement of vectors. Both *Bemisia tabaci* and *Trialeurodes variabilis* whiteflies have been proposed to deleteriously effect papaya crops in Brazil [120]. Thus exposure of healthy papaya plants to PMeV infected *B. tabasi* resulted in the presence of both dsRNA and the sticky disease symptoms [121]. Conversely, *T. variabilis* was unable to transmit PMeV to healthy papaya in plantations in Espírito Santo State under controlled conditions, even when occurrence of the disease was accompanied by high populations of the fly [122]. Thus, additional tests must be performed to discover other potential vectors for PMeV transmission.

PMeV has a broad distribution within papaya-infected plants [123,124]. Since PMeV is closely associated with the papaya latex, tissue damage, a normal occurrence during crop management, could readily result in virus transmission to healthy neighbor plants. Simulating such situations, we evaluated different inoculation methods such as through cuts in leaves and petioles and through friction-induced stem wounds that would be brought about by abrasion with the ladder used for harvesting. Curiously, any wound promoted infection, whereas infection only occurred when diseased latex was directly injected into stem tissue. These results confirmed the suspicions that work tools or tractor movement through the orchard do not transmit PMeV, the protection probably being due to latex polymerization that quickly obstructs the wound surface [125]. Analogous condition occurs with the great majority of plant viruses, supporting the requirement for vector involvement, especially for those vectors possessing piercing-sucking mouthparts.

The early identification of disease symptoms and subsequent eradication of diseased plants is an excellent strategy to control papaya sticky disease [115]. However, an intrinsic disease feature could compromise such action, because the symptoms are displayed only after flowering. Thus, symptom-free infected plants able to transmit PMeV would remain in the field. A molecular diagnostic method was therefore established using the dsRNA molecule as target [124]. This method, based on the occurrence of virus in laticifers-rich tissues [123] and its close association with the latex polymers [126], is applicable for both latex and tissues from asymptomatic plants with the virus detectable fifteen days after inoculation.

Despite different papaya genotypes being evaluated in breeding programs in Brazil, there is still no cultivar resistant to PMeV. We are currently evaluating two different resistance induction strategies in papaya. These are the induction of general pathogen resistance using 'elicitor' molecules of chemical or biological nature, and investigating the functional genomics of PMeV infected papaya. Interesting results have been obtained using nitric oxide (NO) as a chemical elicitor. Seedlings of two different papaya cultivars treated with NO showed an accumulation of compounds used for defense, mainly sugars and phenolics, as well as modification of the transcription patterns of defense genes and a high activity of detoxification enzymes [127]. NO is a signaling molecule involved in several processes such as plant development, induction of genes coding for hormones and proteins related to defense [128]. Thus increased peroxidase (PRO) gene expression and PRO activity were observed, probably to allow the plant to reinforce its cell wall structure and to activate protection-signaling pathways [129]. Similar results were obtained using yeast extracts, indicating that elicitors in these extracts might also enhance the papaya defense response. Experiments are being conducted to evaluate whether the induced defense responses result in papaya protection against PMeV.

Functional genomics is a biotechnology branch concerned with applying the techniques of molecular biology, biochemistry and genetics to analyze the expression and function of

proteins produced under a particular condition. This approach has been used in our laboratory to elucidate the different defense pathways induced in PMeV infected papaya plants. The influence of PMeV on papaya physiology was assessed by determining the main chemical compounds present in the latex, and their relationship with PMeV particles [126]. As the papaya genome has not yet been sequenced, our next objective is to identify the defense genes responsible for resistance against the viral infection. We intend to focus on genes related to RNA silencing pathways, the expression of these genes to be evaluated in plants challenged with viral dsRNA molecules. PMeV dsRNA are present in the latex of infected plants at a high concentration and may easily be extracted [124]. We have also assessed different physical methods, high hydrostatic pressure and mechanical shaking, as well as chemical treatment, denaturating and acid conditions, to disrupt the association between PMeV particles and the latex polymers. Considerable disruption was achieved by shaking and the presence of high urea concentrations. Such preliminary results suggest viable possibilities to obtain PMeV dsRNA molecules that are promising RNA silencing inducers.

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10 Delay in Flowering, Increase in Biomass and Phytoremediation in Genetically Engineered Plants

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Introduction

Increase in crop biomass is very important to the U.S. Government, as it is the goal to produce biofuel ethanol from plant biomass. This is becoming increasingly important because air pollution and global warming are mostly results of burning fossil fuels. In addition, the high costs of fossil fuels have compelled policy makers to encourage scientists to discover alternative energy sources such as lignocellulosic biomass [1,2]. Cellulases are a class of enzymes with great potential for bioconversion of lignocellulosic biomass to ethanol and other important industrial chemicals [3-5]. However, the high costs of cellulase enzyme production in bacterial fermentation tanks are still a barrier to the utilization of these enzymes at the commercial level [6]. Technology to produce hydrolysis enzymes in transgenic crops may become very valuable in reducing these costs [1]. To test whether plants could produce biologically active microbial cellulases, *Arabidopsis thaliana* [7], tobacco, alfalfa and potato [6] have been genetically engineered with a microbial cellulase gene. Also, cellulase-producing transgenic tobacco has been used to test the stability of activity of the heterologues cellulose in plant material after Ammonia Fiber Explosion (AFEX) pretreatment [1].

It is well understood that the biomass production decreases after flowering, the transition from vegetative plant growth (i.e., production of leaves) to a reproductive stage (i.e., production of flowers). Therefore, if the onset of flowering could be delayed, this is assumed to give the plant a longer vegetative growth period resulting in a higher biomass. This key developmental change in the life cycle of the plant [8,9] is controlled by both environmental and developmental signals [10] and has been the subject of many studies, however, it is still not well understood at the molecular level due to the complexity of the flower initiation phenomena [11].

Henderson and Dean [9] recently presented a model that shows the current understanding of flowering in *Arabidopsis*. This model represented the participation of at least eight distinct

pathways regulating the transition from vegetative growth to reproductive organ development. While these pathways perform largely independent of one another, certain interaction takes place among them [11,12]. Several factors, including the light quality, ambient temperature, gibberellins, circadian clock, and photoperiod pathways may promote flowering [9]. Acting against these pathways is the floral repressor gene *FLOWERING LOCUS C* (FLC). A number of genes act to promote FLC expression. It has been shown that FLC is downregulated by vernalization (i.e., long exposure to near-freezing temperatures) and the autonomous pathway genes [9,13-16].

Another concern is the unintended cross pollination of transgenic pollens with other cross breedable crops in the field [17]. Delay in flowering of transgenic crops in the field might avoid or reduce such unintended cross breeding [18].

A third major concern is the presence of high levels of toxic elements such as lead that have accumulated in soil as a result of lead-containing pesticide applications and/or the leakage of underground gasoline storage tanks. The dangers of toxic elements in soil have taken the attention of scientists to study plants that are hyperaccumulators and to understand the basis of this hyperaccumulation [19]. Plants that accumulate lead in their above ground tissues at or above 0.1% on dry biomass basis are lead hyperaccumulators [20].

Here we report the *Agrobacterium*-mediated transformation of the T4 generation of transgenic tobacco constitutively expressing the catalytic domain of E1 endo-1,4-β-glucanase from *Acidothermus callulolyticus* [2] with constitutively regulated FLC. The molecular analysis, delay in flowering time, increase in biomass, and hyperaccumulation of lead by these E1cd-FLC transgenic plants from a Michigan high-lead contaminated soil at the greenhouse level is also presented.

Materials and Methods

Plant Materials

Seeds of T_3 transgenic tobacco (*Nicotiana tabacum* L.) plants expressing E1cd (catalytic domain fragment of E1 endo-1,4-β-glucanase from *Acidothermus callulolyticus*) were used from our previous research [1]. Initially, the T1 seeds were obtained from Dr. Sandra Austin-Phillips of the University of Wisconsin. In their E1cd transformation research, the team used the pZA9 containing E1cd regulated by the CaMV (Cauliflower Mosaic Virus) 35S promoter, the apoplast-targeting leader VSPβ of soybean, and nopaline synthase terminator (Nos); and used *nptII* as the selectable marker gene [2].

Seeds of T_3 plants were washed in water with 0.2% Tween-20 for 10 min and rinsed three times with distilled water, surface sterilized with 70% (v/v) ethanol for 1 min, immersed in 20% (v/v) Clorox (5.25% sodium hypochlorite) for 20 min and then rinsed three times with sterilized double distilled water. Sterile seeds were germinated on Murashige and Skoog [21] (MS) basal medium (Sigma-Aldrich, St. Louis, MO) containing 30 g l^{-1} sucrose and 2.5 g l^{-1} gelrite (Sigma-Aldrich, St. Louis, MO). Cultures were kept under 30 μmol m^{-2} s^{-1} continuous white deluxe fluorescent light at 25 °C. Leaf segments (0.5 cm × 0.5 cm squares) were aseptically excised from the second and third fully expanded *in vitro* produced leaves for infection with *Agrobacterium tumefaciens* [22].

Agrobacterium strain and plasmid

Agrobacterium tumefaciens strain GV 3101 (pMP90RK) [23] containing the 3.232 kb binary vector pGreen [24] was employed for transformation experiments. The plasmid contains FLC from *Arabidopsis thaliana* and phosphoinothricin acetyltransferase gene (*bar*), both under the control of the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (nos) terminator (Figure 1).

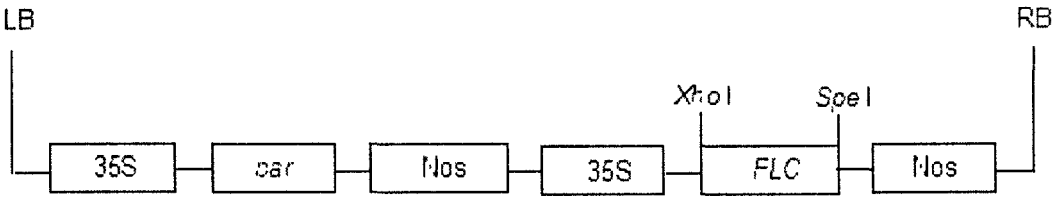

Figure 1. Restriction map of the plasmid pGreen. RB, T-DNA right border; LB, T-DNA left border; FLC, FLC coding region (0.59 kb); 35S, CaMV 35S promoter; bar, phosphinothricin acetyltransferase gene; Nos, nopaline synthase terminator. Plasmid size: about 6 kb. The Agrobacterium containing the transgenes was grown in 10 ml YEP medium (containing 10 g l^{-1} Bacto-peptone. 10 g l^{-1} Bacto yeast extract, 5 g l^{-1} NaCl, pH 7.2) supplemented with 25 g l^{-1} of both kanamycin and gentamycin (25), incubated at 28°C and 250 rpm for 48 h, and the cultures (cell density 0.6-0.8 at A_{600}) were used for transformation.

Agrobacterium-mediated transformation

Leaf segments were infected using the *Agrobacterium* culture at room temperature for 25 min. Then, the leaf explants were blotted on sterilized filter papers and placed upside down on MS medium supplemented with 4.5 μM N^6-benzylamino purine (BAP) and 0.5 μM α-naphthaleneacetic acid (NAA) [6], 30 g l^{-1} sucrose and 2.5 g l^{-1} gelrite (co-cultivation medium). The leaf segments were kept in co-cultivation medium for two days under continuous light as described above for seed culture. Then, they were rinsed three times with sterilized distilled water containing 400 mg l^{-1} carbencillin, blotted onto sterilized filter papers and placed on the same co-cultivation medium supplemented with 400 mg l^{-1} carbencillin and 5 mg l^{-1} glufosinate ammonium for selection of the putative transformants. The produced calli were subcultured in the same medium, and then shoots were excised and rooted on half-strength MS medium containing 400 mg l^{-1} carbencillin and 5 mg l^{-1} glufosinate ammonium in Magenta boxes (Sigma-Aldrich, St. Louis, MO). Well-rooted plantlets were transferred to the greenhouse after acclimatization. Greenhouse conditions were temperature of 25 to 28 °C, 90-95% relative humidity and 190 mmol m^{-2} s^{-1} light.

Polymerase chain reaction (PCR) analysis

After selection on glufosinate ammonium-containing medium, PCR analysis was used to screen the transgenic plants for FLC and E1cd transgene incorporation. Six independent transgenic lines were selected for PCR screening. Total genomic DNA of control and transgenic plants were extracted from leaves as described by Edwards *et al.* [26]. The following set of primers were used: FLC F, 5'-CGA TAA CCT GGT CAA GAT CC-3' (forward primer) and *FLC* R, 5'-

CTG CTC CCA CAT GAT GAT TA-3' (reverse primer), and E1cd F, 5'-GCG GGC GGC GGC TAT TG-3' (forward primer) and E1cd R, 5'-GCC GAC AGG ATC GAA AAT CG-3' (reverse primer). The predicted sizes of the amplified DNA fragments of the transgenes were 338 bp for FLC, and 1.07 kb for E1cd. DNA amplifications were performed in a thermo cycler (Perkin Elmer/Applied Biosystem, Foster City, CA) using REDTaq™ ReadyMix™ PCR Reaction Mix with $MgCl_2$ (Sigma-Aldrich, St. Louis, MO). The PCR profile had an initial denaturation step at 94 °C for 1 min, followed by 30 cycles of 1 min at 94 °C (denaturation), 2 min at 60 °C (annealing) and 3 min at 72 °C (extension). The reaction mixture was loaded directly onto a 1.0 % (w/v) agarose gel, stained with ethidium bromide and visualized with UV light.

RNA-blot Analysis

Total RNA of control plants and PCR-positive transgenic plants for both FLC and E1cd from six putative transgenic lines was isolated from leaves of six-week-old greenhouse plants using the TRI Reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. Twenty micrograms of RNA were fractionated in 1.2% agarose formaldehyde denaturing gel and blotted onto a Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech.) as specified by the manufacturer. The probe was generated by digesting plasmid DNA with *Xho*I and *Spe*I, releasing the 0.59-kb fragment containing the FLC coding region. The digestion reaction mixture was gel-purified using the QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, CA). Probe labeling and transcript detection were obtained using the DIG-High Prime DNA Labeling and Detection Starter Kit II (Kit for chemiluminescent detection with CSPD, Roche Co.) following the manufacturer's protocol.

Flowering Delay, Biomass and Yield Studies

Control and E1cd-FLC transgenic plants were compared concerning plant height, number of leaves produced before flowering, leaf area, days to flowering after transferring to the greenhouse, mean biomass fresh and dry weight, seed yield and thousand seed weight. The experimental design was a completely randomized design (CRD) with four replications. Data were analyzed using MSTAT-C software [27] and means were separated using Tukey's test at the 1 or 5% level.

Segregation Analysis of E1cd-FLC Transgenic Plants

Segregation analysis was conducted using the T_1 generation seeds of the E1cd-FLC self-pollinated plants of the six putative transgenic lines. Forty seeds of each line were cultured on half-strength basal MS medium containing 5 mg l^{-1} glufosinate ammonium. Numbers of germinated and non-germinated seeds were recorded after 2 weeks. The chi square (χ^2) test at $P = 0.01$ was performed to determine if the observed segregation was consistent with a Mendelian ratio.

E1cd Enzymatic Activity of E1cd-FLC Transgenic Plants

Samples of E1cd-FLC and control untransformed plants were assayed for E1 activity as described [1,2]. Briefly, a standard curve was generated using 4 to 160 pmol 4-methylumbelliferone (MU), the product of E1 hydrolization of the substrate 4-methylumbelliferone β-D-cellobioside (MUC). Total soluble protein was isolated from 100 mg fresh leaf tissue using the sodium

acetate grinding buffer and ammonium sulfate precipitation described in Ziegelhoffer *et al.* [6] and quantified by using the BioRad (Hercules, CA) Protein Dye Reagent, measuring the absorbance at 595 nm and comparing the value to the standard curve as specified by the manufacturer. A series of soluble protein dilutions ranging from 10^{-1} to 10^{-3} were made. In a 96-well plate, 1 to 4 µl sample were mixed with 100 µl reaction buffer containing MUC. Plates were covered with adhesive lids and incubated at 65°C for 30 minutes. The reaction was stopped and the fluorescence was read at 465 nm using SPECTRAmax M2 device (Molecular Devices Inc., Sunnyvale, CA) at an excitation wavelength of 360 nm. After subtracting background fluorescence contributed by the control, activity of samples was calculated using the standard curve and compared to the activity of pure E1 reported in Ziegelhoffer *et al.* [6]. Briefly, the nmol MU (from the standard curve) was divided by 30 minutes to calculate nmol MU/min; this number was divided by the µg total protein in the well to calculate the activity.

Vernalization Studies

To test the effect of vernalization on delay in flowering, seeds of control untransformed tobacco plants and seeds of E1cd-FLC tobacco were allowed to germinate on wetted filter papers in petri dishes. Petri dishes were kept in the dark at 4°C [14] for 30 d. Then, the seedlings were planted in the soil and transferred to the greenhouse, where they were grown until flowering.

Phytoremediation Studies

High-lead soil was collected from Reed City in Michigan. The normal soil was prepared similar to the high-lead soil texture but contained only 28.168 PPM lead. The amount of lead in the two soil samples was measured by the A&L Great Lakes Laboratories, Inc., Fort Wayne, Indiana. Seeds of T3 generation of *Ecd*-FLC transgenic and untransformed tobacco plants were germinated in 2" pots either containing high-lead (438.450 mg/Kg) or normal (28.168 mg/Kg) soil to test the possible lead hyperaccumulation by these plants. Four-leaf plantlets were transplanted into 1.5-gallon pots containing the above two soil types. There were four pots per treatment each containing one plant. Three leaves (4th, 5th and 6th leaves from the shoot apical tip) were collected at nine and 12 weeks after transplanting. At the 12 weeks harvest time, stems and roots were also collected for lead accumulation analysis.

To digest the plant material, leaf, stem and root samples were cut in small pieces, and placed in a 104 °C-drying oven over night. The following day the samples were ground in a Cyclotech grinding mill. Then, 0.5 g sample dry matter from each plant was digested with 6 ml of concentrated nitric acid for 30 minutes using an Anton PAAR Multiwave 3000 microwave system. The samples were held at 30 minutes at a pressure of 20 bars and temperature of 180°C, and then allowed to cool. Each of the cooled samples was filtered into a tared vessel, and brought to 50 g final weight with deionized water. In order to keep the dissolved solids low, each sample was further diluted 1:20 with deionized water.

To measure the accumulated lead in each sample, a method was used by A&L Great Lakes Laboratories Inc., Fort Wayne, Indiana, where ions are produced by a radio frequency inductively coupled plasma using a Perkin Elmer DRC Plus ICP-MS. Using this system, the analyte species in a liquid digest were nebulized and the aerosol transported by argon gas into a plasma torch. The ions produced are entrained in the plasma gas and introduced by means of an interface

into a mass spectrometer. Then, the ions produced are sorted according to their mass to charge ratio and quantified with a channel electron multiplier. Statistical analysis was performed as indicated in the '*Flowering delay, biomass and yield studies*' section above.

Results and Discussion

Polymerase Chain Reaction

PCR analysis showed the integration of both FLC and E1cd in all the lines. The E1cd transgenic control plants showed only presence of E1cd (data not shown).

RNA-blot Analysis

All the transgenic lines showed high levels of FLC mRNA transcripts (Figure 2). No band was detected for control plants. Based on PCR and RNA-blot analyses, transformation and selection efficiency can be estimated as 100%.

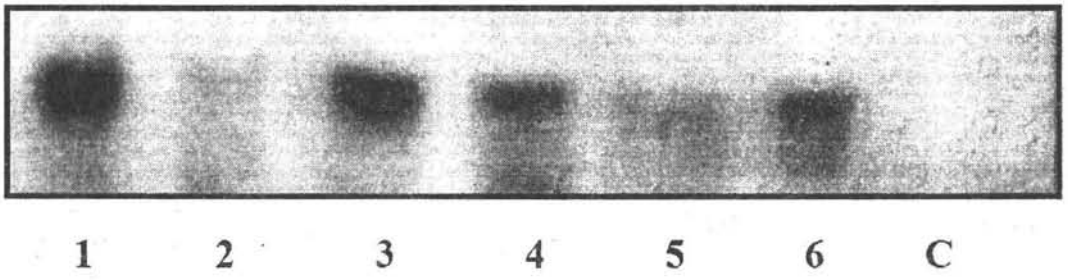

Fig. 2. RNA-blot analysis of FLC in E1cd-FLC transgenic plants. Lanes: 1 to 6 = E1cd-FLC transgenic lines; C = E1cd transgenic control.

Flowering Delay, Biomass and Yield Studies

Control plants flowered 23 days after transfer to the greenhouse (Table 1). Transgenic plants showed 32 to 44 days of vegetative growth before to switching to the reproductive stage, after transfer to the greenhouse (Table 1). Therefore, transgenic plants showed a delay in flowering of 9 to 21 days, with a mean of 15 days greater control plants (Table 1). This is more or less consistent with the previous results in *Arabidopsis* [11,13,14,29], *Brassica napus* [30] rice [31] and our previous study on tobacco [18]. In the previous study, transgenic lines 1 and 4 showed the greatest delays in flowering, 18 to 25 days, with a mean of 21 days, and 17 to 26 days, with a mean of 20 days, respectively [18]. FLC is known to prevent premature flowering in *Arabidopsis* [13,14,28,29], *Brassica napus* [30] rice [14] and tobacco [18]. In our experiment, all transgenic lines were shorter than control at flowering time, with no significant difference in nodes or leaf number (Table 1; Plate I). Practically speaking, the shorter stem might be one of the advantages of biomass plants such as maize considering normal lodging or stem breakage in the field. Lines 1 and 4 produced leaves significantly larger than control and other lines (Table 1; Plate I).

Lines 1 and 4 had significantly more biomass fresh weight than all four other lines ($P \leq 0.01$) and the control plants ($P \leq 0.05$) (Table 2). Biomass dry weight was more or less the same in control and all the transgenic lines (Table 2).

Table 1. Comparison between control and E1cd-FLC transgenic tobacco plants in flowering delay and vegetative growth before flowering†.

Plants	*Days to flowering after transferring to the greenhouse*	*Flowering delay (d)*	*Number of leaves produced before flowering*	*Leaf area (cm^2) flowering time (cm)*	*Plant height at*
Control	23b	0b	20a	333.5c	113.3a
Transgenic Lines					
Line 1	44a	21a	19a	518.0a	50.0b
Line 2	39a	16a	20a	218.5e	56.8b
Line 3	32a,b	9a,b	19a	345.8c	45.5b
Line 4	43a	20a	19a	477.5b	47.0b
Line 5	38b	15a	21a	239.5d,e	48.0b
Line 6	35a,b	12a,b	20a	253.0d	54.5b
Overall mean of six lines	38	15	20	342	50

†Measured with second fully expanded leaf from the bottom. In each column, means followed by the same letters are not significantly different using Tukey's test at $P \leq 0.01$.

Table 2. Differences between control and E1cd-FLC transgenic tobacco plants in biomass, thousand seed weight and seed yield per plant†.

Plants	*Biomass FW/plant (g)*	*Biomass DW/plant (g)*	*Thousand seed weight (mg)*	*Seed yield/plant (g)*
Control	187.0a,b,c	32.75a	658c	5.51a,b,c
Transgenic Lines				
Line 1	275.3a,b	29.25a,b	701bc	4.90b,c,d
Line 2	181.8b,c	30.50a,b	740a,b	3.87c,d
Line 3	164.3c	21.00b	792a	3.75d
Line 4	291.3a*	35.50a	745a,b	6.89a
Line 5	159.8c	19.25b	726b	4.01c,d
Line 6	179.5b,c	27.00a,b	588d	6.30a,b
Overall mean of six lines	209	27	715	5

†In each column, means followed by the same letters are not significantly different using Tukey's test at $P \leq 0.01$; *Significantly different from control using Tukey's test at $P \leq 0.05$.

Plate I. E1cd-FLC transgenic tobacco (line 1) plant (right) compared to control E1cd plant (left); Note the short stem and larger leaves of transgenic plant.

Except line 3, seed yield per plant was not significantly different between control and transgenic plants. Also, with exception of line 6, the seed yield i.e. thousand seed weight of all transgenic lines was significantly higher than the control plants (Table 2). Line 3 produced the lowest seed yield but the seeds were larger and heavier (Table 2). In transgenic rice, FLC

caused reduced fertility and even sterility, suggesting that expression of FLC could get in the way with other elements of reproductive developments [31], that we believe has been due to the transgene position effect rather than the transgene physiological effect. In our case, except line 3, FLC did not reduce the fertility.

Expression of *bar* gene in the T_1 progeny

Seeds of all the E1cd-FLC transgenic lines were germinated on selection medium with a segregation ratio of 3:1 (Table 3). None of the control non-transformed seeds germinated on the selection medium.

Table 3. Segregation of glufosinate ammonium resistance (germinated vs. non-germinated seeds) in E1cd-FLC T_1 progeny†.

Lines	*Number of germinated seeds*	*Number of non-germinated seeds*	*Expected ratio*	χ^2
1	30	10	3:1	0.000^{ns}
2	27	13	3:1	1.999^{ns}
3	30	10	3:1	0.000^{ns}
4	32	8	3:1	0.533^{ns}
5	31	9	3:1	0.133^{ns}
6	29	11	3:1	0.133^{ns}

†Forty seeds were used for each line; ^{ns}Non-significant.

Enzymatic activity of E1cd in E1cd-FLC transgenic plants

According to Ziegelhoffer *et al.* [2], E1 in E1cd plants hydrolyzed 4-methylumbelliferone β-D-cellobioside (MUC) to 4-methylumbelliferone (MU) at a rate of 40 nmol of substrate per microgram per minute. The enzymatic activity of E1 enzyme extracted from apoplast-targeted transgenic *E1cd* which were further transformed with FLC (so called E1cdFLC) was 1.4726 nM/μg/min. This activity is similar to the E1 enzymatic activity that was originally reported by Ziegelhoffer *et al.* [2] and confirmed by Teymouri *et al.* [1] for earlier E1cd transgenic generations of these plants. This confirms that addition of FLC does not affect E1cd enzymatic activity.

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Vernalization studies

In *Arabidopsis*, vernalization has the ability to down-regulate FLC expression [9,13-16]. Tobacco is an annual and a warm-season crop, which normally does not require vernalization to induce flowering. As expected, our results indicated that vernalization had no effect on flowering time, which is consistent with a similar experiment in *Brassica napus* [30]. Also, vernalization is downstream of FLC, and because the CaMV35S promoter used in pGreen construct is a strong promoter, it would have probably overturned any effects vernalization may have had on FLC [30], and therefore transgenic tobacco plants were not cold-responsive.

Phytoremediation studies

The results of the phytoremediation studies are shown in Table 4. According to Baker *et al.* [19], the lead hyperaccumulators are plants that accumulate lead in their above ground tissues at and/or above 0.1% on dry biomass basis. Table 4 shows that tobacco plant is apparently a

Table 4. Lead hyperaccumulation in leaves, stems and roots of transgenic versus non-transformed control tobacco plants grown in normal versus high-lead soil types for 9-week and 12-week†.

Factors		*Normal soil*		*High-lead soil*	
		9-week after planting	*12-week after planting (harvest time)*	*9-week after planting*	*12-week after planting (harvest time)*
Pb in Leaves (mg kg^{-1} DW)	Control Plants	2.4 ± 0.15a	3.9 ± 1.87a	17.5 ± 0.69a	7.8 ± 1.34a
	E1cd-FLC Plants	3.0 ± 1.46a	1.8 ± 0.24a	30.8 ± 3.16b	10.0 ± 0.82b
Pb in Stems (mg kg^{-1} DW)	Control Plants	-	0.5 ± 0.03a	-	17.3 ± 1.44a
	E1cd-FLC Plants	-	0.7 ± 0.25a	-	29.5 ± 4.42b
Pb in Roots (mg kg^{-1} DW)	Control Plants	-	3.1 ± 0.74a	-	182.4 ± 4.3a
	E1cd-FLC Plants	-	2.6 ± 0.44a	-	170.4 ± 4.7a

†Numbers within each soil type followed by the same letter are not significantly different from each other ($p<0.05$).

hyperaccumulator of lead, as in our experiment control tobacco samples whether grown in low-lead or high-lead soil accumulated lead above this amount. Our results also show that the E1cd-FLC transgenic plant leaves and stems accumulated lead from the high-lead soil significantly more than the control untransformed plants. Overall roots accumulated much more lead than leaves and stems when plants were grown in high-lead soil. Also, the accumulation of lead in leaves, stems or roots of control untransformed in low-lead soil were not significantly different than those from transgenic plants. Although it must be tested in the future, we do not believe that the E1cd transgene alone had any role in hyperaccumulation of lead from high-lead soil. However, clearly, the E1cd-FLC played a very important role in this

hyperaccumulation. This significant increase in lead uptake by E1cd-FLC might be due to the increase in biomass resulting from delay in flowering of the E1cd-FLC transgenic plants.

Acknowledgements

The authors wish to thank Dr. Richard M. Amasino (Department of Biochemistry, University of Wisconsin, WI) for providing the pGreen, Dr. S. Austin-Phillips (University of Wisconsin-Madison, Madison, WI) for the seeds of E1cd tobacco, and the A&L Great Lakes Laboratories Inc. for the lead analysis of soil and plants. This research was supported under DOE STTR Grant DE-FG02-04ER86183 to Edenspace Systems Corporation, the MSU Project GREEEN, The MSU Research Excellent Funds (REF) and the Consortium for Plant Biotechnology Research (CPBR).

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11 The Lesion Mimic Mutants as a Tool for Unveiling the Gene Network Operating During Biotic and Abiotic Plant Stresses

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Introduction

Programmed cell death (PCD) is a process of cell suicide central to the development, homeostasis and integrity of multicellular organisms [1]. In plants, PCD plays a role in resistance against pathogens [2]. Two types of cell death result when plants are attacked by a pathogen: the hypersensitive response (HR), a form of PCD, and a progressive form of cell death (necrosis), a symptom of the disease. HR is explained by the gene-for-gene resistance model and requires the interaction between an avirulence gene (*Avr*) of the pathogen and the resistance gene (*R*) of the plant [3]. HR protects the plant both by trapping the parasite in the dead cells and by providing a signal to neighboring healthy cells to create an anti-pathogen environment [4]. The interplay via HR between host and pathogen leads to the formation of small lesions mainly on the leaves. The events that take place in the plant cell undergoing HR are: the change of the membrane potential and ion permeability of the plasma membrane (increase in extra-cellular pH and K^+, Ca^{2+} and H^+ influx into cell), oxidative burst generation through reactive oxygen species (ROS), mainly superoxide anions, hydrogen peroxide, and hydroxyl radicals [4]. ROS cause lipid peroxidation, partially responsible for cell membrane disfunctioning, and play a role as direct antimicrobials, promoting cross-linking of cell-wall proteins and/or acting as a signal for inducing further gene expression [5]. Phenolics, phytoalexins and other compounds are synthesized and callose, lignin, hydroxyproline-rich glycoprotein are deposited at the cell wall. Furthermore, HR may generate a signal that activates host defense mechanisms inducing long-lasting systemic acquired resistance (SAR) that includes synthesis of pathogen related (PR) proteins and a complex network leading to accumulation of signalling molecules (mainly salicylic acid, SA; ethylene, ET; jasmonic acid, JA) conferring resistance to a broad spectrum of pathogens [6]. Recent studies propose the ubiquitin/26S proteasome complex degradative capacity central in the signalling processes [7].

In plants, a high number of spontaneous and induced mutant phenotypes, dominant or recessive, as well as of transgenic origin, are characterized by spontaneous formation on leaves of discrete or expanding lesions of variable size, shape and colour [8]. Because such spontaneously occurring lesions resemble those resulting from the plant-pathogen interaction, the mutants have been called lesion mimic mutants (LMMs) [9].

A first classification of LMMs considers the pathways involved in the HR driven mechanisms controlling cell death, PCD initiation and PCD suppression. Lesions, which in the affected cells result from infection, injury or mutation, initiate in response to the production of a toxic metabolite, which autocatalytically propagates through the surrounding cells. Lesion development is eventually arrested by a suppression system contributing to the formation of lesions with characteristic size and appearance. According to this model, LMMs are classified initiation/determinate or propagative/feedback lesion mimics [10]. Both classes of LMMs represent a useful tool for understanding how cell death is regulated and executed in plants [11-13].

The isolation of lesion mimic (LM)-related genes and their functional analysis attribute the LM phenotype not only to the alteration of the signalling defense pathway but also to additional physiological alterations, like the blockage of metabolic processes, involvement of the ubiquitin system and energy overproduction [14,15]. These physiological alterations are internal- (developmental stage and genetic background) and external- (abiotic stresses, among which light intensity, temperature, drought, wound, chemical) –dependent processes. In addition, in a number of cases a cross-talk between the two pathways has been observed [8] and free radicals production seems to connect the two processes.

The present review is an updated analysis of LMMs in different plant species, focusing on the action of the genes involved in the signalling network leading to the LM phenotype.

Occurrence of Lesion Mimic Mutants in Plant Species

In maize the observation of mutants which spontaneous necroses independent of pathogen attack dates back to the '20s [16]. Only in the '90s however the studies of LMMs had a burst that have made them a powerful tool for studying the cell death control also in relation to the plant-pathogen interaction (Figure 1). Rice, *Arabidopsis* and maize are the species with the

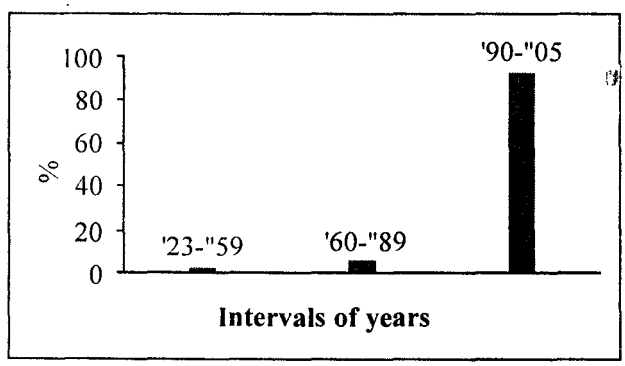

Figure 1. Percentage of LMM described and/or studied in three different year intervals from 1923 to 2005.

highest percentage (ca. 27%) of described LMMs. In Table 1-3, are reported the LMMs identified and/or studied in several species, including, when known, responsible gene, triggering stress, disease resistance, putative gene function and literature references.

Maize

Several disease LMMs have been studied in maize since the first described by Emerson [17-21]. In 1975, Neuffer and Calvert identified two dominant disease-lesion mimic mutants, lesion-1 (*Les1*) and lesion-2 (*Les2*) and called them 'lesion mimic'. Other authors have termed similar mutants as 'necrotic', 'auto-necrotic' or 'blotched'.

The recovery of maize *Rpl* alleles with LM phenotype provided a direct evidence that at least some LMMs are variants of race-specific resistance genes, and suggested that misregulation of resistance gene function may lead to cell death [22].

Table 1. Lesion mimic mutants identified in maize

Gene	*Triggering stress*	*Resistance to pathogens*	*Putative gene function*	*References*
Les1-Les8 *(Lesion mimic)*				[11]
Les9		*Bipolaris maydis*		[25,26]
Les10-Les17				[2]
Les22	Background, light, developmental stage		Uroporphyrogen decarboxylase (UROD)	[24]
les23	Background, light			[27]
Les -D101				[11]
Les-EC91				[11]
Les-MA102				[11]
Les -1369				[11]
Les-28Q				[11]
lls1 *(lethal leaf spot)*	Background, light, developmental stage, wound, pathogen	*Cochliobolus heterostrophus* *Puccinia sorghi*	Wild type (WT): pheophorbide *a* oxygenase	[15,28-30]
les-35587				[11]
les-911				[11]
les-1790				[11]
rp1-NC				[11]
les-MO141				[11]
les-2013				[11]
les-2014				[11]
les-A467				[11]
Rp1		*P. sorghi*		[31]

Table 2. Lesion mimic mutants identified in rice

Gene	*Triggering stress*	*Resistance to pathogens*	*Putative gene function*	*References*
sl (sekiguchi lesion)				[42-44]
cdr1 (cell death and resistance)	Developmental stage	*Magnaporthe grisea* when lesion are still formed		[45]
cdr2	Developmental stage	*M. grisea* when lesion are still formed		[45]
Cdr3	Developmental stage	*M. grisea* before the lesion are formed		[45]
spl1 (spotted leaf)		*M. grisea*		[49]
spl2-spl4				[49]
spl5		*M. grisea*		[49]
spl6				[49]
spl7	High temperature or UV solar radiation		WT: heat stress transcriptor factor (HSF)	[52]
spl9				[49]
spl11		*M grisea*	U-box/ARM repeat protein	[51,49]
		Xanthomonas oryzae pv. *oryzae*		
spl5-2		*M. grisea* *X. oryzae* pv. *oryzae*		[50]
spl12		*M. grisea* *X oryzae* pv. *oryzae*		[50]
spl13-spl14		*M. grisea* *X. oryzae* pv. *oryzae*		[50]
spl15		*M. grisea* *X oryzae* pv. *oryzae*		[50]
blm (blast lesion mimic)		Long-day condition Temperature shift from 28 to 24 °C		[46,47]
lrd27-lrd44 (lesion resembling disease)	Light	*lrd37* and *lrd40* *X oryzae* pv. *oryzae*		[53]
ebr3 (enhanced blast resistance)	*X oryzae* pv. *oryzae*, *M. grisea* Mock-inoculation	*X. oryzae* pv. *oryzae*		[54]
M. grisea necr1 (necrosis in rice)	Developmental stage	*M. grisea* when lesion are still formed		[50]

Table 3. Lesion mimic mutants identified in *Arabidopsis*

Gene	*Triggering stress*	*Resistance to pathogens*	*Putative gene function*	*References*
1	*2*	*3*	*4*	*5*
acd1 (*accelerated cell death*)	Light		WT pheophorbide *a* oxygenase	[67.15]
acd2	Light		WT red chlorophyll catabolite reductase	[14]
acd5	Development stage. *P. syringae* inoculation		WT ceramide kinase	[68.69]
acd6		*P. syringae*	WT protein with an N-terminal ankyrin repeat domain a C-terminal and transmembrane domain	[70.71]
acd11			WT glycolipid transfer protein	[72]
agd2 (*aberrant growth and death*)		*P. syringae*	WT. amino acid-derived molecule that promotes development and suppresses defenses	[73.74]
cet mutants (*constitutive expression of the thionin gene*)			*cet* genes act within JA- and SA-dependent signalling cascades	[75.76]
cpn1 (*copine1*)	Low-humidity Low-temperature	*P. syringae* *Peronospora parasitica*	Copine protein	[77]
cpr5 (*constitutive pathogen response*)		*P. parasitica*	WT: novel membrane integrated protein	[78]
cpr20 ,cpr21		*P. syringae* *P parasitica*		[79]
cpr22	Low-humidity	*P. parasitica*		[80]
dll1 (*disease-like lesions1*)	Long-day growth. high humidity			[81]
dnd1				

1	2	3	4	5
(defense no death)		Fungi, bacteria, viruses	WT: cyclic nucleotide-gated ion channel (CNGC2)	[82,83]
dnd2			WT: cyclic nucleotide-gated ion channel (CNGC4)	[84]
Y23		*P syringae*		[85]
hlm1 *(HR-like lesion mimic)*		*P. syringae*	WT: cyclic nucleotide-gated ion channel (CNGC4)	[86]
hrl1 *(hypersensitive response-like lesions)*	*P. syringae* *P. parasitica*			[87]
len1 *(lesion initiation 1)*	Short-day condition		WT: chloroplast chaperonin (Cpn60â)	[65.88]
lin2 *(lesion initiation 2)*	Developmental stage, light		WT: coproporphyrinogen III oxidase	[89]
lsd1 *(lesions simulating disease)*	Long day		Zinc finger protein	[90,91]
lsd2-lsd5	Developmental stage, long-short day	*P. parasitica*		[90,92]
lsd6, lsd7		*P. syringae*		[93,94]
mod1 *(mosaic death1)*			WT: enoyl-ACP reductase	[66]
ssi1 *(suppressor of, SA-insensitivity)*	*P. syringae* *P. parasitica*		WT: negative regulator of a number of SA-dependent processes	[93,95]
ssi2	Cucumber mosaic virus		WT: plastid-localized stearoyl-ACP desaturase	[96]
ssi4	Developmental stage, moderate humidity	*P. syringae* *P. parasitica*	WT: *TIR-NBS-LRR* type *R* gene	[97,98]
vad1 *(vascular associated death1)*	Light intensity	*P. syringae*	WT: membrane protein containing a GRAM domain	[62]

Other maize LMMs do not seem to involve genes whose function is related to pathogen recognition or response. This is the case of *lls1* where the lesions first appear near the tip of the oldest leaf and then gradually move downwards to its base; this pattern is progressively repeated on every leaf up the plant [23]. Lesions propagation in *lls1* implies a defect in a mechanism containing cell death. Besides, when the *lls1* leaf enters a senescence stage, wound or pathogen attack trigger typical lesion formation. In absence of an external stress, 'spontaneous' lesions may reflect an endogenous stress due to the accumulation of a phototoxic metabolite. Recently, Pruzinskà *et al.* [15] reported that *Lls1* encodes the pheophorbide *a* oxygenase (PaO) enzyme, which catalyzes the removal of the highly phototoxic chlorophyll pheophorbide *a* catabolite. Such an evidence explains the formation of light-dependent lesions and the loss of chloroplast ultrastructure observed in the *lls1* mutant [23]. *Les22* is a dominant LM; as for *lls1*, the onset of lesions is developmentally regulated and seems dictated by an age-gradient, independent from chloroplast. Hu *et al.* [23], cloned the gene mutated in *Les22*, and found that it encodes UROD, the fifth enzyme in the C-5 porphyrin pathway, important in the production of both chlorophyll and heme in plants, involving consequently the activity of catalases, heme-requiring enzymes. The dominance of this mutation indicates in *Les22* an example of an inborn error of metabolism and that this disorder be a mutation due not to a gain of a new function but rather to a null mutation in one copy of the *urod* gene [24].

Tomato

In 1948, Langford [32] studied the leaf autonecrosis phenomenon following the introgression of *Cladosporium fulvum* (leaf mold) resistance genes from *Solanum pimpinellifolium* into *S. esculentum*. He verified that necrosis was consequent to the combination of the *Cf2* gene from *S. pimpinellifolium* with a "chromosome complex" (*ne*) derived from the cultivated tomato, under certain environmental conditions. Subsequently, genetic and molecular investigations proved that the *Rcr3* gene is specifically required for achieving the *Cf2*-mediated resistance [33]. Kruger *et al.* [34] isolated *Rcr3* and found that it encodes a secreted papain-like cysteine endoprotease, and that $Rcr3^{pim}$ (the *Ne* allele from *S. pimpinellifolium*) is needed for the expression of *Cf*-2 resistance by suppressing $Rcr3^{esc}$-dependent autonecroses (*ne*, from *S. esculentum*).

Recently, Santangelo *et al.* [35] described a tomato plant (V20368) with necrotic leaf spots mimicking disease lesions that was singled out in a progeny under selection in Moscow (breeding material of Ignatova Svetlana). At increasing temperature and high light intensity, the progeny exhibited necrotic lesions on the leaves, with acropetal progression (autonecrosis) similar to the case previously described by Langford. The same authors characterized the V20368 line and confirmed that autonecrosis was caused by the interaction of genes *Cf-2* and $Rcr3^{esc}$. At the conditions described, this interaction triggers an oxidative burst, as evident from a strong increase in H_2O_2 production and in catalase, peroxidase and ATPase activities [35]. In addition, by grafting the necrotic mutant on the cv. Riogrande and *vice versa*, the authors proved that the acropetal progression of necroses was not due to a movement of a signal molecule, since the necroses never appeared on both the parts of the grafted plant but only on the autonecrotic one (scion or understock) (Plate I). Finally, the chlorotic and necrotic spots appearing on V20368 plants after organophosphoric insecticide Fenthion treatment reveale i a

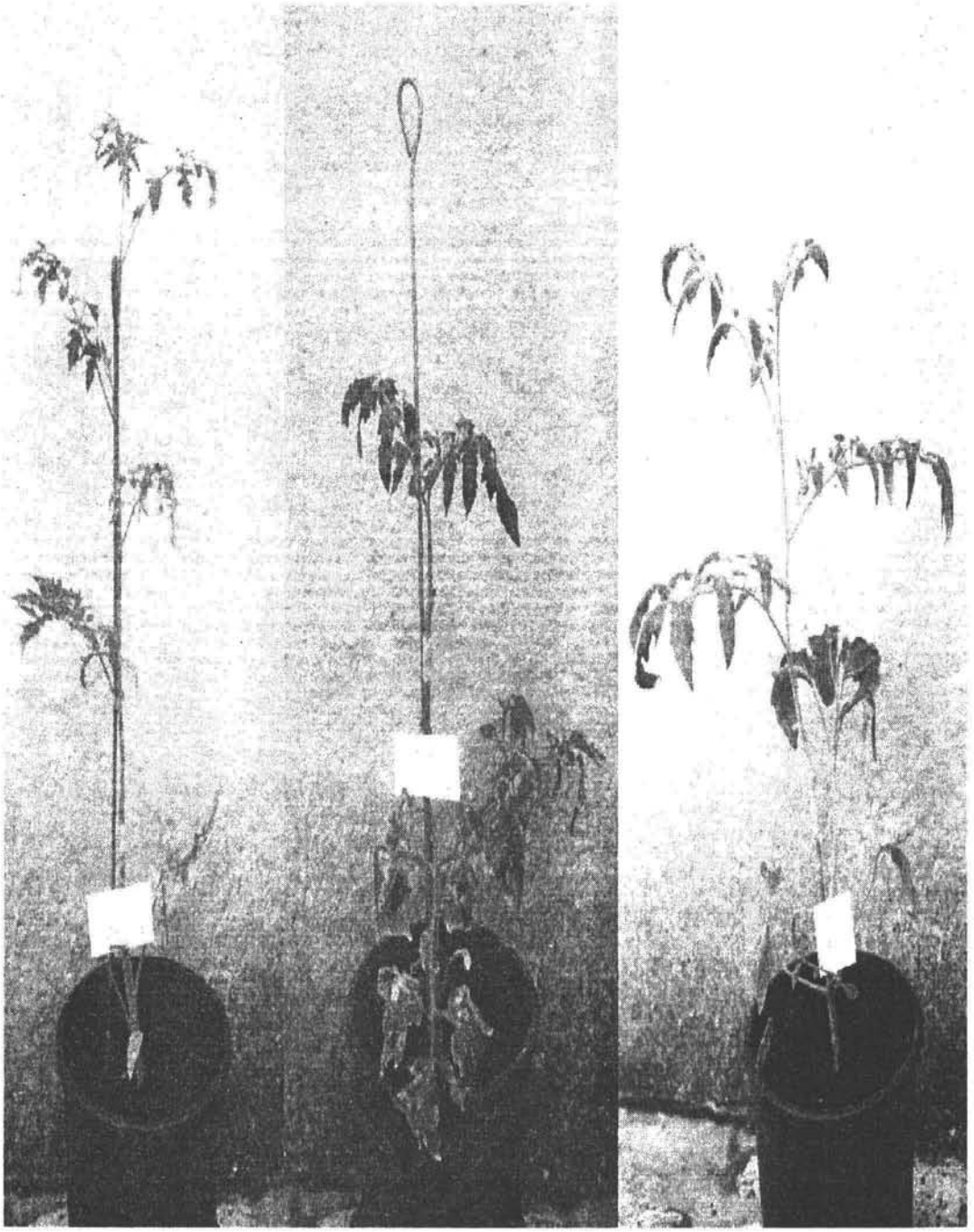

Plate I. Tomato grafted plants with visible necrotic spots on self grafted V20368 (left), on V20368 when rootstock (middle) or scion (not shown) and lacking on the Riogrande (RIG) WT when scion (middle) or rootstock (not shown) and on self-grafted RIG (right).

low threshold of stress perception in the absence of the *Fen* gene which is responsible for the necrotic spots appearing on leaves sprayed with Fenthion.

In the *Cf2* mediated resistance, the requirement of a *Rcr3* encoded cysteine protease, a key enzyme in animal and plant PCD-apoptosis [36,37], may imply a link between developmental cell death control and pathogen resistance. Recently, Rooney *et al.* [38], found that Avr2 binds and inhibits Rcr3 and propose that the Rcr3-Avr2 complex enables the Cf2 protein to activate HR by a conformational change of the protein structure. They assume that Cf2 recognizes both $Rcr3^{pim}$ bound with Avr2 and unbound $Rcr3^{esc}$ because of a conformational change in the protein ($Rcr3^{esc}$ differs from $Rcr3^{pim}$ in one amino acid deletion and six amino acid substitutions), which mimics the $Rcr3^{pim}$-Avr2 complex.

Other tomato mutants, radiation or chemically induced and having a necrotic phenotype are: *ne*-2 (*necrosis*-2), which exhibits weak small plants, with many greyish necrotic spots [39]; *mcn* (*maculonecrotic*) with virescent-yellow leaves turning necrotic centrally [40]; *mgn* (*marginal necrotic*), showing reduced plant height with yellowish leaf margins becoming necrotic [40]; *m-2* (*mottled-2*), temperature sensitive, with many fine chlorotic spots on the leaves [41].

Rice

The first rice LMM identified was the Sekiguchi lesion mutant (*sl*) in which a tight linkage between the LM gene and the blast resistence *Pi-ta* gene was observed [42,43]. Afterwards, Marchetti [44] studied the *sl* spontaneous mutation in two American rice lines in which lesion formation was induced by *Bipolaris oryzae* and *Magnaporthe grisea* and by certain chemical agents. Other spontaneous or mutagenized LMMs in rice were subsequently described. Most of these mutants showed a significant resistance to the blast fungus *M. grisea* (*cdr1*, *cdr2*, *Cdr3*, *blm*) and a high induction of defense-related genes (*PBZ1*, *PR1*), together with high level of phytoalexin production (momilactone A, sakuranetin), deposition of autofluorescent compounds as callose [45-47]. Moreover, proteome analysis of *cdr2* revealed a high metabolic activity during PCD in this LMM [48]. The largest class of rice LMMs includes *spl* mutants, some of which are of spontaneous origin [49,50] and have enhanced resistance to both fungal and bacterial pathogens. Some of the *spl* mutations are probably allelic to *cdr* [49]. The most studied LMM in rice is *spl11*, isolated in an EMS mutagenized population. The SPL11 protein contains a U-box-domain and an armadillo (ARM) domain, suggesting a role of ubiquitination system in the control of plant cell death [51].

Barley

In barley, Wolter *et al.* [55] described a set of *mlo* mutants, all alleles of the same genetic locus. To date 17 molecularly characterized *mlo* mutants are described [56] which possess strong resistance to *Blumeria graminis* f. sp. *hordei* isolates, through complex of phenolics, callose and proteins formed at the cell wall near the penetration site of the biotrophic parasite. The *mlo*-dependent resistance requires *Ror1* and *Ror2* [57]. *Mlo* mutants, raised in conditions of low temperature, show spontaneous manifestation of lesions that mimic a pathogen attack. The *Mlo* gene encodes the prototype of a family of heptahelical integral membrane proteins unique to plants [58]. The spontaneous cell death in *mlo* mutants suggests that Mlo functions

as a negative regulator of cell death and senescence. The two allelic, recessive mutations at the *nec1* locus - *nec1a*, induced by gamma-ray or by diethyl sulfate treatment [59], and *nec1c*, of spontaneous origin [60] - result in appearance of dark brown necrotic spots on green tissues visually resembling HR. Recently, Rostoks [61] by studying some Fast Neutron induced mutants, which have an extensive deletion in the *nec1* gene, confirmed the orthology of the *Arabidopsis HLN1* gene, that encodes the cyclic nucleotide-gated ion channel 4 (CNGC4), to the barley *NEC1* gene. As in other LMMs, the *nec1* mutants have increased expression of PR genes (*HvPR-Ia* and β-1,3-glucanase).

Arabidopsis

In *Arabidopsis*, a wide range of LMMs exists and the isolation of different genes underlying the necrotic phenotype reveals the complexity of the pathways responsible for the LM phenomenon [8]. A new LMM named *vad1* was identified by Lorrain *et al.* [62] in a population of *Arabidopsis* mutagenized by T-DNA insertion [63]. This mutant exhibits propagative HR-like lesions along the vascular system and displays increased resistance to virulent and avirulent strains of *Pseudomonas syringae* pv. *tomato*. In *vad1* both cell death and disease resistance depend on SA production. The gene encodes a plant membrane protein containing a GRAM domain, an intracellular protein binding or lipid binding signalling domain important in membrane-associated processes [64]. VAD1 may play a role in defense and cell death signalling associated with the cell membrane or act as a cell death regulator. Ishikawa [65] described the *len1* mutant in which the lesion phenotype appears only under short-day conditions. The study suggests that, as in other *Arabidopsis* LMMs (*acd1*, *acd2* and some *lsd*), the chlorophyll breakdown is involved with the tetrapyrrole metabolism affected in *len1*. The *mod1* mutant is sensitive to temperature, displaying deficiency in fatty acid biosynthesis. In continuous light it shows the characteristic LM phenotype at 26°C. In addition the mutation indicates that a deficiency in fatty acid biosynthesis has pleiotropic effects on plant growth and development [66]. For this reason this mutant can be included in the LM class.

Transgene-Induced Lesion Mimic Mutants

The mechanisms responsible for the LM phenotype can be studied based on transgenic plants which allow to improve knowledge on PCD. In fact, some transgene-induced LM are altered in the defense response or in the cellular homeostasis pathways.

A classification of transgenic LMMs has been reported by Mittler and Rizhsky [99] who subdivided them in to four classes: pathogen-derived, signal transduction-inducing, general metabolism perturbing and killer genes (Table 4).

In tomato, *nahG* transgene, expressing salicylate hydroxylase, an enzyme that degrades SA to catechol [100], has necrotic lesions under high light conditions. Likewise tomato plants overexpressing the *Pto* gene show an LM phenotype. Tang *et al.* [101] hypothesize two possible mechanisms linking *Pto* overexpression to ROS production: the reaching of sublethal ROS concentration due to the photosynthetic process and to the *Pto* overexpression; inhibition of ROS scavenging enzymes, probably originated from increased SA levels, which negatively interact with peroxidases and catalases. Intriguingly, the double transgenic *nahG/Pto* tomato plants have not an LM phenotype. These results allow to conclude that the signalling pathways activated by *Pto* overexpression suppress the cellular damage caused by SA depletion [102].

Table 4. Classification of lesion mimic transgenes according to Mittler and Rizhsky [99]

Transgene	*Source*	*Function*	*Defense*
Class A. Pathogen-derived genes			
TMVcp (*tobacco mosaic virus coat protein*)	TMV (N')	Avr elicitor	NT
Avr9	*C fulvum*	Avr elicitor	Yes
Elicitin	*P. cryptogea*	Avr elicitor	Yes
AvrRpt2	*P. syringeae*	Avr elicitor	Yes
Class B. Signal transduction-inducing genes			
bO (*bacterio-opsin*)	*H. halobium*	Proton pump	Yes
Cholera toxin	*V. cholerae*	Inhibit GTPase	Yes
sGTP- BP (*small GTP-binding protein*)	Plant	GTP-binding protein	Yes
Antisense *CAT* (*catalase*)	Plant	removal of ROI (reactive oxygen intermediates)	Yes
Antisense *APX* (*ascorbate peroxidase*)	Plant	removal of ROI	NT
Antisense *PPO* (*protoporphyrinogen oxidase*)	Plant	heme biosynthesis	Yes
Class C. General metabolism-perturbing genes			
Invertase	Yeast	hexose transport	Yes
Hexokinase	Plant	hexose metabolism	Yes
CaMV gVI (*cauliflower mosaic virus*)	CaMV	inclusion body protein	Yes
rPS14	Plant	ribosomal protein	NT
Class D. Killer genes			
Barnase	*B. amylolique-faciens*	RNase	Yes
DTA (*diphtheria toxin A subunit*)	*D. pertussis*	inhibits translation	NT
Protease-related (Class B/C/D)			
Ubiquitin	Plant	protein degradation	Yes
Kunitz-type trypsin inhibitor	Plant	protein degradation	NT

Abbreviation: NT, not tested

The involvement of the SA pathway in lesion formation is conditioned by the genetic background of the LMM. In *Arabidopsis*, this is evident when crossing the *nahG* transgenic line with different LMMs: only in some cases the suppression of spontaneous lesion formation is observed, as outlined later in this review.

Some transgenic LMMs are related to signal transduction-inducing genes. The introduction of *bO* (a gene from *Halobacterium halobium*, encoding the bacterio-opsin protein) in tobacco, tomato [103] and poplar [104], results in transgenic plants resembling dominant initiation LMMs. Pontier *et al.* [103] proposed a model of the possible action of bO in tobacco plants: the plasma membrane (PM)-localised bO proton pump acts in transgenic plants as a passive proton channel and translocates protons that are pumped to the apoplast by a PM-localised H+-ATPase. The enhanced flow of protons into the cytosol activates the HR signal transduction pathway and results in induction of PCD, SA biosynthesis, PR gene expression and SAR establishment, including resistance to pathogens [105]. In poplar, however, the susceptibility to pathogens was unaffected by *bO* expression. This fact may indicate a different reaction to *bO* expression in trees and herbaceous species [104]. A further step has been made considering wound-inducible *bO*-transgenic plants that normally do not exhibit LM phenotype. When wounded, these genotypes have an activated SAR with consequent resistance to pathogens [106].

In *Oryza sativa*, the transgenic expression of *OsRac1*, encoding a small GTP-binding protein, in the LM mutant *sl* resulted in suppression of ROS generation and cell death induced by the rice blast fungus and calyculin A, a protein phosphatase I inhibitor [107].

The transgenosis offers a very useful tool to investigate host-pathogen interaction, PCD phenomena and the related metabolic alterations. The transgenic LM lines obtained in the last years are listed in Table 5.

What can we Learn from Lesion Mimic Mutants

The LMMs identified over the years have provided the best evidence for the existence of genes regulating PCD in plants. Accordingly, two groups of mutants can be distinguished: with mutation in genes playing a direct role in maintaining cellular homeostasis; with mutations controlling cell death in the host-pathogen interaction. Whatever the mutation triggering the LM phenotype might be, a set of signal molecules is commonly operating, making evident the sharing of certain steps between the two groups and therefore the existence of a 'conservative' activation of cell death.

LMMs Phenocopying Metabolism Misregulation

Examples of malfunctioning of the chlorophyll biosynthesis inducing an LM phenotype are described in maize and *Arabidopsis*. In the former, *Les22* encodes UROD, an enzyme involved in the tetrapyrrole biosynthesis, important for the production of both chlorophyll and heme. The mutant, characterized by light-dependent minute necrotic spots on leaves in a specific plant stage [24], is phenotypically and genetically similar to *'porphyria cutanea tarda'* the human metabolic disorder which manifests itself as light-induced skin morbidity due to an excess of photoexcitable uroporphyrin accumulation. The leaf tissue damage is the result of both the accumulation of a photoreactive urophorphyrinogen intermediate and of the lack of

Table 5. Additional up-dated list of transgene-induced LMMs and LM lines following cross with transgenic mutants

Transgene	*Source*	*Function*	*Defense*	*Reference*
Rice				
NH1 *(NPR1 homolog 1)*	Plant	Regulator of SAR		[108]
OsLSD1	Plant	Negative regulation of PCD and positive regulation of callus differentiation		[109]
AtNPR1 *(non expressor of PR1)*	Plant	Role in plant stress response	*X. oryzae* pv. *oryzae*	[110]
Gns1 *(1,4-glucanase)*	Plant			[111]
Arabidopsis				
VWA *(von Willebrand A domain)*	Plant	Interaction with BON1/CPN1 protein function	*P. syringae* pv. *tomato*	[112]
FPS1S *(farnesyl diphosphate synthase isoform 1S)*	Plant	Putative regulatory role in the MVA pathway		[113]
Tobacco				
PAB1 *(Polyadenylate-binding proteins)*	Yeast	Gene expression TMV	*P. syringae* pv. *tabaci* *P. tabacina*	[114]
Tomato				
bO *(bacterio-opsin)*	*H. hal* *obium*	Proton pump	*P. syringae* pv. *tomato*	[106]

ROS degradation, particularly of H_2O_2, by catalase and peroxidase. An *Arabidopsis* mutant related to chlorophyll biosynthesis disorder is *lin2,* which shows small spots or stripes of collapsed tissue, both on siliques and leaves, under long day conditions. Ishikawa *et al.* [89] found that *LIN2* encodes a coproporphyrinogen III oxidase, the enzyme operating in the same pathway soon after UROD.

The chlorophyll disruption is an event common to senescence and defense responses in plants. Because of this, the LMMs with phenotype closely related to the breakdown of the chlorophyll are of particular interest. The interruption of the chlorophyll degradation pathway

can in fact trigger the production of extremely phototoxic compounds able to absorb light and donate active electrons to molecular oxygen, leading to toxic free radicals that act as cellular signals. Mutations in this pathway block the activity of the pheophorbide *a* oxygenase and the red chlorophyll catabolyte reductase (RCCR) evidenced in the mutants *acd1* and *acd2* of *Arabidopsis* [15,14] and in *lls1* ACD1 homologue of maize [23]. An elegant proof of the role of *LLS1* is provided by the study of Spassieva and Hille [115] that used the tomato *LLS1* homologue in virus induced gene silencing (VIGS) in order to obtain a light-dependent phenotype resembling the *lls1* mutant of maize. This resulted in a LM phenotype, both in mono (maize) and dicotyledons (tomato, tobacco), suggesting a functional conservation of this function across different species. As previously mentioned, *len1* is a loss of function mutant in the biosynthesis of the chloroplast chaperonin 60β, a protein involved in chlorophyll breakdown. The mutant displays an LM phenotype as a result of accumulation of phototoxic catabolic compounds [65]. In the light-dependent *les23* LM of maize, Penning *et al.* [27] succeeded in suppressing lesion formation, possibly by removing free radicals, through a system involving the *slm1* factor (*suppressor of lesion mimic 1*).

A metabolic pathway affecting cellular homeostasis when studied in LMMs is fatty acid biosynthesis. In plant cells, most fatty acids are found in lipid forms, such as diacylglycerol, sphingomyelin, and ceramide, which function either as essential components of cell membranes or as important regulators of cell growth, differentiation, secretion and apoptosis [116]. Mutations affecting key steps in the lipid biosynthesis pathway were described in *Arabidopsis*. In *mod1* plants, a reduction of enoyl-ACP reductase, a component of a fatty acid synthesis multifunctional protein complex, affects the total lipid content and the functionality of cell membranes. The disruption of cellular integrity triggers both the death process and the release of signalling molecules inducing cell death in the surrounding cells [66]. *ssi2* entails a defect of a stearoyl-ACP desaturase (S-ACP DES) which catalyzes the first step in the pathway from stearic to linolenic acid, a precursor of JA. The protein regulates the levels of unsaturated fatty acids (FAs) in cells [117]. Consequently, some JA-dependent responses are impaired, the case of *Botrytis cinerea* resistance and *PDF1.2* gene expression. The authors cited conclude that modification of the ratio saturated/unsaturated FAs or changes in their subcellular distribution might regulate a cross-talk between defense signalling (SA and JA) pathways, by altering protein phosphatase activity. This in turn might lead to the stimulation of protein kinase- or to the inhibition of mitogen activated protein kinase (MAPK) pathways.

Bioactive lipids as ceramides and their related sphingolipids, structural components of cell membrane, have a role as second messengers in animals, affecting cell fate, by eliciting apoptosis and/or altering differentiation or cell-cycle events [118]. Liang *et al.* [69] isolated in *Arabidopsis* the *ACD5* gene that encodes a lipid kinase (CERK) with *in vitro* specificity for short-chain ceramides, postulating the same role *in vivo*. Because the *acd5* LMM accumulates ceramides, and late in development is characterized by spontaneous cell death, the authors suggest that in the wild type, phosphorylation of ceramides dampens directly the proapoptotic effect of unphosphorylated ceramides.

The active role of sphingosine transfer protein in plant PCD control has been studied by Brodersen *et al.* [72]. The *acd11* mutant, identified among stable lines generated by insertion of a modified maize *Ds* element, developed chlorotic leaf margins at the two-to six-leaf stage. *ACD11* encodes a homolog of mammalian glycolipid transfer protein and has sphingosine

transfer activity *in vitro*, thus providing a link between *ACD11* function and sphingolipid metabolism as well as a clue that also sphingolipid signalling is used to regulate PCD and defense in plants.

LMMs Phenocopying Pathogen Disease Response

HR, characterized by a local resistance reaction after pathogen recognition, involves a rapid change in ion flux (K^+ and Cl^-) across the plasma membrane, an increase in Ca^{2+} concentration and acidification of the cytosol, activation of protein kinases, phosphatases, phospholipases, local accumulation of SA and ROS [119,120]. The final outcome is a rapid cell death around the site of infection, which stops pathogen spreading. Many LMMs, that present constitutive expression of defense mechanism, represent interruptions of key steps in the signalling pathway leading to HR.

Ion fluxes are required for the activation of MAPKs specific to defense responses, and yet the increment in Ca^{2+} influx and levels are prerequisites for the mounting of the oxidative burst following plant-pathogen recognition. The *dnd1*, *hlm1* and *cpn1 Arabidopsis* LM phenotypes are attributed to mutations encoding Ca-related proteins [83,77,86]. The first two mutations encode a cyclic nucleotide-gated channel (CNGC), respectively CNGC2 and CNGC4, closely related; the third one encodes a copine, a Ca-dependent phospholipid binding protein. *dnd1* is classified as a rare conditional LM, sometime forming pinpoint lesions associated with growth of young plants at low light intensity and relatively low humidity. These mutants show constitutive activation of defense mechanisms and cause perturbation of cell homeostasis pinpointing the importance of these channels in the absence of pathogen attack and in the signal transduction leading to the defense process upon infection.

It has been speculated that ROS involved in activating the HR may be generated by NADPH oxidase, an enzyme constituted (in mammalian neutrophil) by membrane and cytosol proteins and by a GTP-binding protein (Rac) with a pivotal role for the functionality of such a complex. Kawasaki *et al.* [107] showed the involvement of NADPH oxidase as an activator of ROS for HR induction. Transgenic expression in the rice *sl* LMM of a dominant-negative variant of *OsRac1* (a small GTP-binding protein) resulted in suppression of ROS generation and cell death induction by a rice blast fungus and calyculin A, a protein phosphatase 1 inhibitor.

In *Arabidopsis*, the *lsd1* mutant represents a clear example of the complex interplay between plant responses to pathogen attack and environmental factors (*i.e.*, light intensity) linked to ROS production. The *lsd1* mutant phenotype was initially described as a superoxide-dependent with chlorotic/necrotic lesions under long (16 h) or continuous photoperiods or after infection by an avirulent pathogen [90,121]. Later genetic studies revealed that *LSD1*, encoding for a zinc-finger protein, regulates catalase expression and consequently stomata conductance, thus displaying a role in excess excitation energy (EEE) dissipation during the photorespiration process [122]. LSD1 is also supposed to be a component of a SA-dependent signalling pathway for a CuZnSOD activation scavenging superoxide ion [123]. In this sense, *lsd1* behaves similarly to a catalase-deficient plant, resulting unable to detoxify ROS (H_2O_2 and superoxide) produced either during hypersensitive cell death response or failing to dissipate EEE effectively. As reported for *ssi2*, a LMM phenocopying metabolism misregulation, where a link between the pathogen response pathway and the correct functioning of cellular metabolism is evident, the

study of *lsd1* mutant has pointed out the roles of LSD1 in both light acclimation and restricting pathogen-induced cell death.

Interestingly, some mutants with constitutive high SA levels, defense-gene expression, and disease resistance also display an LM phenotype. The *Arabidopsis* transgenic plant expressing the NahG bacterial enzyme, impairing SA accumulation [124], is a powerful tool for unveiling SA action. Crosses of the *nahG* transgenic line with different *Arabidopsis* LMMs can lead to suppression (*lsd6, lsd7, acd5, acd11, cet3, cpr22, ssl, dll1*) or to no effect (*cet2, cet4.1, lsd2, lsd4, lsd5, agd2*) on spontaneous lesion formation. In the first case, the role of SA is crucial, while in the latter the SA accumulation occurs downstream the formation of lesions. Crosses with the *npr1* (*non expressor of PR1*) mutant are also informative on the SA role [8]. Mutants with defects in *NPR1* fail to respond to various SAR-inducing treatments, displaying little expression of *P*R genes and exhibiting increased susceptibility to infections [125]. These mutants accumulate SA after infection and likely NPR1 functions downstream to SA and upstream to *PR* gene expression [126]. The most important outcome from the study of these double mutants is the evidence of the existence of a SA-dependent NPR-independent pathway, as well as the function of SA and NPR1 in the amplification and/or in the correct timing of lesions initiation.

SA is not the only signalling compound produced by plant in response to pathogen attack. JA and ET are certainly implicated in the response to biotic and abiotic stresses, but their interaction appears differently governed under different stresses and pathogen attack. Until now, the involvement of JA and ET in the signalling pathway has been studied following the expression of two genes: *PDF1.2*, a marker of the ET and JA pathway activation, and *Thi 2.1* (thionin), which has a JA-directed expression. The *cpr5* mutation shows *PDF1.2* expression. When this gene is introduced into an ethylene-impaired mutant line, the appearance of lesions is delayed suggesting that ET plays a role in the proper timing and amplification of cell death [8]. The *cpr5* and *cpr6* mutants, that produce constitutively high levels of SA, both express SA- and JA-dependent marker genes, also exhibiting increased resistance to virulent *P. syringae* and *P. parasitica* strains [127]. When the two mutants are crossed with *npr1*, in the double mutants the expression of *PR1* (a marker gene of the SA-dependent SAR in *Arabidopsis*) and *PDF1.2* remains at the same high constitutive level of the *cpr6* mutant, while PR1 is suppressed in *cpr5* [128]. *cpr5* and *cpr6* regulate the resistance through distinct pathways, while SA-mediated NPR1-independent resistance works in combination with components of the JA/ET–mediated response pathways [129].

The work carried out on the *cet* mutants [75], which overproduce JA and its bioactive precursors, supports the existence of two distinct defense response pathways, SA- and JA-dependent, that are turned on in response to pathogen invasion.

Recently Tang *et al.* [130] have noted that the *edr1* mutant displays enhanced stress responses and spontaneous necrotic lesions under drought conditions in the absence of pathogen, suggesting that EDR1 (encoding a *CTR1*-like kinase and functioning as a negative regulator of disease resistance and ET-induced senescence) is also involved in stress response signalling and cell death regulation. Double mutant analysis revealed that these drought-induced phenotypes require the SA but not the ET signalling pathways.

What Else the Lesion Mimic Mutants can Teach us

Although LMMs represent usually an alteration of a single gene, their phenotype is influenced by a number of external (temperature, light, photoperiod, drought, humidity) and internal (plant age and genetic background) factors. Since several LMMs under certain environmental conditions display microscopic lesions, they reveal a connection between defense and stress response. Interestingly, several of these mutants also have defects related to plant development, indicating a relationship between defense induction and growth disorders.

Beside a deep dissection of the early events in the host-pathogen interaction and of the SA role—also in relation with other plant hormones—LMMs are useful for the analysis of new metabolic pathways or of the relevance of key compounds. Pathways associated to a direct or indirect role on PCD induction through the study of LMMs or related transgenics, in *Arabidopsis* are the ones leading to isochorismate-derived compounds of *acd11* [131], the tetrapyrrole metabolism of *len1* [65], the mevalonic acid pathway by farnesyl diphosphate synthase isoform 1S (FPS1S) overexpression.

The complexity of the cell death (CD) process in plants entails, in cases, the action and involvement of nitric oxide (NO), mitochondria and caspases. While for NO most of the available information derives from pharmacological studies using NO scavengers and NO synthase inhibitors [132], an involvement of phosphorylation of proteins targeted to mitochondria has been found by Takahashi *et al.* [133] through the use of the *cdr* rice LMMs. It is important to stress that mitochondria and chloroplasts, organelles with highly oxidizing metabolic activities and sustained electron flows, are the main sites of ROS production, therefore undoubtedly interact in the CD machinery. Mittler and Rizhsky [99] reported that the transgenic expression of a modified ubiquitin gene unable to polymerize (an essential step in protein degradation) and of a 'Kunitz'-type trypsin inhibitor, induced a LM phenotype. More recently, Zeng *et al.* [51] by using the *spotted leaf11* (*spl11*) rice mutant—displaying a spontaneous cell death phenotype and enhanced resistance to rice fungal and bacterial pathogens—isolated the *Spl11* gene and demonstrated that the predicted SPL11 protein contains both a U-box domain and an armadillo repeat domain, both involved in ubiquitination and protein–protein interactions in yeast and mammalian systems. Ubiquitination is one of the key regulatory mechanisms of apoptosis in animals and should play an important role in plant cell death and pathogen defense.

Arabidopsis is a species where the high number of LMMs supported an extensive analysis of this phenomenon. In other species the availability of such mutants is increasing as in rice, with the *spl* series [49,51], the *cdr1* and *cdr2* [133] and the recent *blm* [47]. Also in a species like groundnut, LMMs have been recognized [134]. These authors, through induced mutagenesis and *in vitro* culture, obtained two identical mutants, allelic for the disease lesion mimic leaf trait.

The study of the mechanisms underlying CD has taken advantage of the progress in the genome and proteome studies. Starting from the analysis of physiological and biochemical alterations induced by a disorder in the cellular homeostasis of defense signalling, the dissection of the mechanisms of CD through the use of LMMs is now benefited by the microarray technique [72], proteome analysis [48], EST sequencing [135], virus induced gene silencing and ectopic expression of single domain [115]. Additional data are acquired resorting to classical breeding. Penning *et al.* [27] aiming to associate maize LM *les23* to quantitative trait loci (QTL) able to

modify the level of lesion expression, analyzed an F_2 population derived from the cross between *les23* and the Mo20W inbred line. QTL analysis identified one major factor, designated *slm1*, controlling the majority of the variation in lesion initiation timing, and suggested that the initiation time of lesion is a determining factor for final lesion severity. Because other QTLs affected the severity of lesion expression without modifying the lesion initiation date, the authors speculated that a dramatic change in the lesion phenotype can be controlled by a single major QTL.

Langford about 60 years ago attributed a potential evolutive meaning to the autonecrosis phenomenon observed in a tomato breeding program for *C. fulvum* immunity involving genes from a wild species. Such a prescient hypothesis has acquired present interest on the basis of genetic, molecular and biochemical evidences in prokaryotes and eukaryotes. According to Ameisen [1] the regulation of the premature cell death (cell suicide) must have represented one of the driving forces in the evolution towards biosystem complexity. This is supported by the parallel existence of different death programs and by the fact that gene products and molecular pathways participating in the premature CD may play a crucial role for the cell survival. Each cell of a given genome experiences both competition and cooperation between different genetic modules. Moreover, in the plant cell the nuclear, mitochondrial and plastidial genomes coexist and interact. Not only nuclear genes are governing plastidial and mitochondrial gene expression, but also signals originating in the plastids and mitochondria may act in reciprocal events, following the modifications elicited by the environment. Mitochondria are supposed to derive from ancient bacterial origin representing the outcome of an initial symbiosis between the primitive aerobic bacteria and the eukaryotic cell. The PCD is seen by Ameisen [1] as a residual of the 'initial, evolutive struggle' between these two kinds of ancestors. The development and survival of the eukaryotic cells should have been dependent on the cellular homeostasis supported by the interaction of the mitochondria and the eukaryotic cell, as regulated by PCD.

In such a view, the different LM mutants offer themselves as a powerful research tool for deeping the knowledge of cell death expression and control, as well in clarifying the role of genetic diversification and speciation in the evolution of living organisms from prokaryotes to eukaryotes. Such a kind of mutants also allow advances for a better understanding of the metabolic pathways interconnecting in plants nuclear, mitochondrial and plastidial genes in response to biotic and abiotic stresses.

Finally, further interest in LMMs arises in space flights experiment planning. In such microgravity conditions, photosynthetic processes are modified, thus altering the perception and response to biotic and abiotic stresses. To dissect out and critically analyze the defense response components of the plant-pathogen interaction under spaceflight conditions, Leach *et al.* [136] developed a model system based on rice LMMs that makes them suitable for experiments on Space Station so minimizing the manipulation needed by the Mission Specialists when phytopathogenic microrganisms are involved.

Acknowledgements

We thank Andrea Mazzucato for critical reading of the manuscript and Francesco Salamini for his helpful comments.

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12 Changes in Seed Vigor and Reactive Oxygen Species during Accelerated Ageing of Guar Seeds

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Introduction

Seed deterioration leads to reductions in seed quality, performance, and seedling establishment. Seed deterioration is due in part to the reason of membrane lipid peroxidation and leakiness caused by reactive oxygen species (ROS) attacking [3, 24], including surperoxide radical ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$) and other organic radical [19]. At the cellular level, the excess production of ROS causes cell death [25]. Changes in protein structure and nucleic acid damage can also attribute to ROS attacking [13]. ROS scavenging enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and glutathione reductase (GR) participate metabolism of the ROS, and they inhibit and reduce the damage [11, 13, 20, 26].

The processes by which seeds die during storage has received considerable attention in the literature [26], these processes include decline in fatty acid content [17, 28], decrease in non-enzymatic antioxidant level [3, 17] and ROS scavenging enzyme activity [2, 21, 23], and increase in malondialdehyde (MDA) level [2, 23].

The loss in seed viability during storage is a gradual process, are subjected to influence of seed water content and storage temperature [5]. Accelerated ageing of seeds, seed lot was exposed to high temperature and high relative humidity (RH), leads to the loss of vigor and eventual viability, and is an excellent method to assess the changes in seed vigor during storage.

Cyamopsis tetragonoloba (L.) Taubert is a plant of *Cyamopsis* of Leguminosae, originally grows in Africa or India [27]. Guar seed is a very important industrial material for petroleum, paper making and food [10]. Zheng *et al.* [29] studied the effects of temperature on germination, they reported that the optimum temperature for seed germination is 25-30°C according to germination percentage of seeds only, but the germination percentage of seeds is 64% at 25°C, and 72% at 30°C. $CaCl_2$ and glycerol can increase germination rate and germination index of guar seeds [30]. To our knowledge, ageing and accelerated ageing of guar seeds, effects of

ROS and their scavenging enzymes on seed vigor have up to date not been studied. In this paper, guar seeds were used as experimental material, accelerated ageing of seeds was used to simulate or to substitute the natural ageing, relationships among seed vigor, production and scavenging enzyme activities of ROS during accelerated ageing were studied.

Materials and Methods

Plant Material

Guar (*Cyamopsis tetragonoloba* (L). Taubert) seeds were collected at maturity from plant growing in Xishuangbanna Tropical Botanical Garden of the Chinese Academy of Sciences, Mengla, Yunnan of China. After drying to about 0.12 g H_2O/g DW by air at 25±2°C, seeds were kept at 15°C until used.

Accelerated ageing Treatment of Seeds

Seeds were placed into a nylon mesh bag, and then suspended in a closed desiccator (Φ=22 cm), and were subjected to accelerated ageing at 40°C and 100% RH for 0, 3, 6, 9, 12 and 15 d, respectively.

Determination of Water Content

Water content of seeds was determined gravimetrically (103°C for 18 h). 30 seeds were sampled for each determination. Water content of seeds is expressed on a dry mass basis [g H_2O (g dry mass)$^{-1}$; g/g].

Germination Test

Batches of 50 seeds were germinated on two filter paper and 15 ml deionised water in Petri dishes (Φ=12 cm) at 15, 20, 25, 30, 35 and 40°C, respectively, in the dark for 5 days. Seeds showing radicle emergence were scored as germinated. Fresh weight of seedlings produced by germinating seeds does not include cotyledons.

Determination of Superoxide Radical and Hydrogen Peroxide

$\cdot O_2^-$ was measured as described by Elstner and Heupel [7] by monitoring the nitrite formation from hydroxylamine in the presence of $\cdot O_2^-$, modified as follows. Seeds accelerated aged for different time were homogenized in 6 ml of ice-cold 50 mM sodium phosphate buffer (pH 7.8) at 4°C, and the brei was centrifuged at 12 000 *g* for 10 min. The supernatant was used for determination of $\cdot O_2^-$. The reaction mixture contained 0.9 ml of 50 mM phosphate buffer (pH 7.8), 0.1 ml of 10 mM hydroxylamine hydrochloride, and 1 ml of the supernatant was incubated at 25°C for 20 min, and then 0.5 ml of 17 mM sulfanilamide and 0.5 ml of 7 mM naphthylamine were added to the reaction mixture. After incubation at 25°C for 20 min, the absorption in the aqueous solution was read at 530 nm. A standard curve with nitrite was used to calculate the production rate of $\cdot O_2^-$ from the chemical reaction of $\cdot O_2^-$ and hydroxylamine.

The content of H_2O_2 was measured by monitoring the absorption of titanium-peroxide complex at 410 nm according to the method of MacNevin and Uron [12] and Partterson *et al.* [16], modified as follows. Seeds were homogenized in 6 ml of 5% (w/v) trichloroacetic acid, and then centrifuged at 12 000 *g* for 10 min. After 1 ml supernatant and, 9 ml of 0.2% (w/v) titanium tetrachloride hydrochloride solution were mixed, the absorbance of reaction solution was measured at 410 nm using H_2O_2 as a standard.

Assay of SOD

Seeds accelerated aged for different time were ground with a mortar and pestle at 4°C in an extraction mixture composed of 50 mM phosphate buffer (pH 7.0), 1.0 mM EDTA, 0.05% (v/v) Triton X-100, 2% (w/v) PVPP and 1 mM AsA. The homogenate was centrifuged at 16,000g for 15 min, after which the supernatant was transferred to a new tube and kept at -0°C.

Assay of SOD (EC 1.15.1.1) activity was based on the method of Beauchamp and Fridovich [4], who measure inhibition of the photochemical reduction of NBT at 560 nm, modified as follows. 1 ml reaction mixture contained 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 75 μM NBT, 16.7 μM riboflavin and enzyme extract (ca 50 μg protein). Riboflavin was added at last and the reaction was initiated by placing the tubes under two 9-W fluorescent lamps. The reaction was terminated after 15 min by removal from the light source. An illuminated blank without protein gave the maximum reduction of NBT, and therefore, the maximum absorbance at 560 nm. SOD activity (mean of five replicates) is presented as absorbance of sample divided by absorbance of blank, giving the % of inhibition. In this assay, 1 unit of SOD is defined as the amount required to inhibit the photoreduction of NBT by 50%. The specific activity of SOD was expressed as unit mg^{-1} protein.

Assays of APX and CAT

Accelerated aged seeds were ground with a mortar and pestle at 4°C in 5 ml of 50 mM Tris-HCl (pH 7.0), containing 20% (v/v) glycerol, 1 mM AsA, 1 mM dithiothreitol, 1 mM EDTA, 1 mM GSH, 5 mM $MgCl_2$ and 1% (w/v) PVPP. After two centrifugation steps (12.000g for 6 min and 26,900g for 16 min, respectively), the supernatant was stored at -20°C for later determinations of enzyme activities of APX and CAT.

APX (EC 1.11.1.7) was assayed as the decrease in absorbance at 290 nm due to AsA oxidation, by the method of Nakano and Asada [15]. The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 1 mM AsA, 2.5 mM H_2O_2 and enzyme source (ca 50 μg protein) in a final volume of 1 ml at 25°C.

CAT (EC 1.11.1.6) activity was determined by directly measuring the decomposition of H_2O_2 at 240 nm as described by Aebi [1], in 50 mM potassium phosphate (pH 7.0), containing 10 mM H_2O_2 and enzyme source (ca 50 mg protein) in a final volume of 1 ml at 25°C.

Lipid Peroxidation Product

Lipid peroxidation product was determined as the concentration of TBA-reactive products, equated with MDA, by the method of Hodge *et al.* [9]. MDA content was calculated according to C_{MDA} (nmol L^{-1})=$6.45\times[(A_{532}-A_{600})-0.0571\times(A_{450}-A_{600})]$, and was expressed as nmol mg^{-1} protein.

Protein Assay

Protein was measured following the procedure of Bradford [6], using BSA as a standard.

Statistical Analysis

The data of changes in water content, leakage rate, germination percentages, fresh weight of seedling produced by germinating guar seeds were analyzed using a one way ANOVA model from the SPSS 11.0 package for Windows (SPSS Inc.).

Results

Effects of temperature on germination percentage and germination rate of guar seeds

Final germination percentage of guar seeds and fresh weight of seedling produced by germinating seeds increased with increasing germination temperature until 30°C, and then decreased (Figure 1). The temperature at which 50% of seeds germination (GT_{50}) was about 18°C; and the optimum germination temperature, as measured by germination percentage and fresh weight of seedling after 5 d of imbibition, was 30°C (Figure 1a; Table 1). The time required for 50% of seeds germinated at 20, 25, 30, 35 and 40°C was approximately 53, 35, 20, 20 and 20 h, respectively (Figure 1).

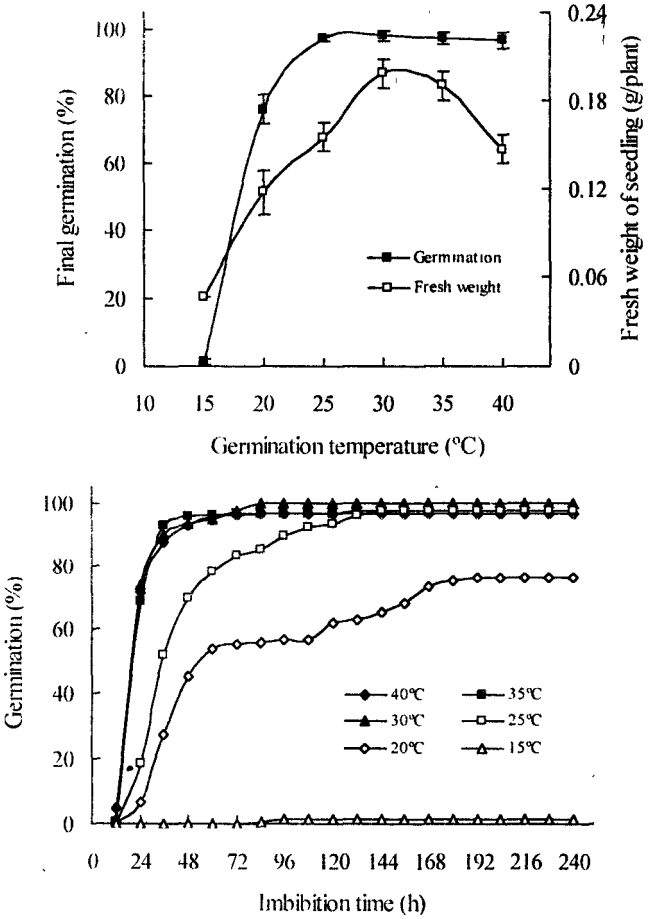

Figure 1. Effect of temperature on final germination percentage, fresh weight of seedling (a), and germination rate (b) of Cyamopsis tetragonoloba seeds. Seeds were germinated at indicated temperature in the dark for 5 d. Seeds showing radicle emergence were scored as germinated. Fresh weight of seedling produced by germinating seeds does not include cotyledons. All values are means ± SD of three replicates of 50 seeds each.

Table 1. Effects of temperature on final germination percentage and fresh weight of seedling of *Cyamopsis tetragonoloba* seeds. All values are mean ± SD of three replicates of 50 seeds each, and are statistical results of a one-way ANOVA. d.f., degrees of freedom; MS, mean squares

Germination temperature (℃)	*15*	*20*	*25*	*30*	*35*	*40*	*d.f.*	*MS*	*F-rations*	*P-value*
Germination (%)	1.33±0.470	76.0±4.320	97.33±0.940	100.0±0.000	97.33±1.870	96.67±2.490	5	4412.490	1241.01	0.000
Fresh weight of seedling (g/plant)	0.047±0.000	0.117±0.019	0.155±0.010	0.198±0.012	0.190±0.009	0.147±0.010	5	0.012	97.87	0.000

Changes in Water Content and Leakage Rate of Seeds

Water content of dried guar seeds was originally 0.124±0.001 g/g, and increased gradually with increasing accelerated ageing (40°C, 100% RH); and water content of seeds accelerated aged for 15 d increased by 353% than that of non-aged seeds (Figure 2a; Table 2).

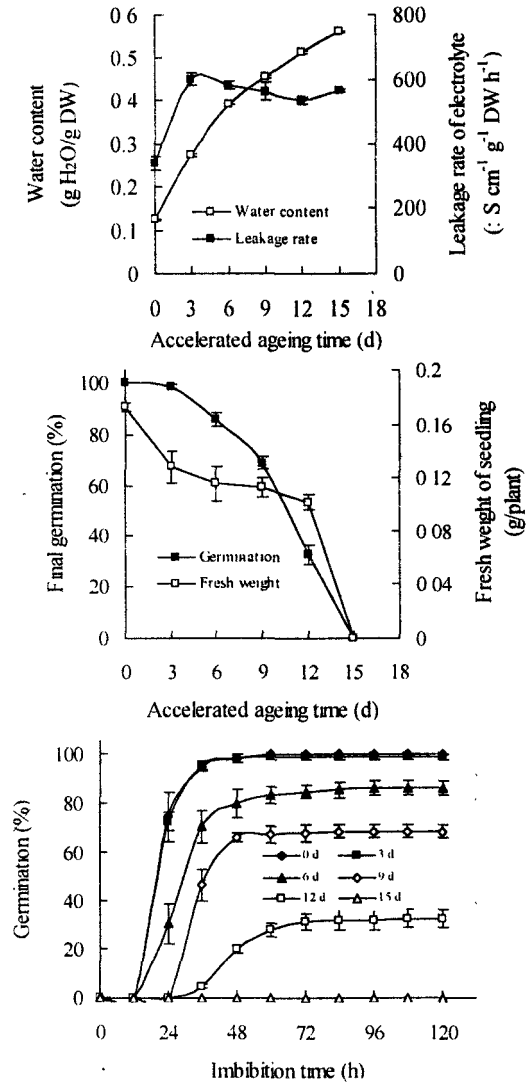

Figure 2. Changes in water content, leakage rate (a), final germination percentage, fresh weight of seedling (b), and germination rate (c) during accelerated ageing of Cyamopsis tetragonoloba seeds. After seeds were accelerated aged at 40°C and 100% RH for indicated time, water content, leakage rate and germination of seeds were determined as described in Materials and Methods. Seeds showing radicle emergence were scored as germinated. Fresh weight of seedling produced by germinating seeds does not include cotyledons. The values are means ± SD of five replicates of 10 seeds each for determination of leakage rate, the others are means ± SD of three replicates of 50 seeds each.

Table 2. Changes in water content, leakage rate of electrolyte, germination percentage and fresh weight of seedling during accelerated ageing of guar seeds. The values are mean ± SD of five replicates of 10 seeds each (for leakage rate) and three replicates of 50 seeds each (for others), and are statistical results of a one-way ANOVA. d.f., degrees of freedom; MS, mean squares

Accelerated ageing time (d)	*0*	*3*	*6*	*9*	*12*	15	d.f	MS	F-ratios	*P-value*
Water content (g H_2O/g DW)	0 124± 0 001	0.273± 0.004	0.393± 0.000	0.456± 0 003	0 513± 0 003	0.560± 0.003	5	0 080	8419.30	0.0
Leakage rate ($\mu S\ cm^{-1}g^{-1}DW\ h^{-1}$)	338.50± 21 860	622.40± 19 910	582.00± 14.470	562.05±27 290	523.24±10 820	536.83±4.780	5	33617.930	76.88	0.0
Germination (%)	100±0 000	98 67± 0 940	86 0± 2 830	68 67± 2.490	32 67± 3 770	0.0±0 000	5	4797 160	634.92	0.0
Fresh weight of seedling (g/plant)	0 173± 0 003	0 128± 0 012	0.116± 0.016	0.113± 0 007	0 112± 0 006	0.0±0.000	5	0 0120	96.26	0.0

Leakage rate of electrolyte of seeds rapidly increased during the initial stage of accelerated ageing, and then slowly decreased; but the leakage rates were still much higher than those of non-aged seeds, for example, the leakage rate of seeds accelerated aged for 15 d increased by 66% compared with non-aged seeds. It was noted that leakage rate of seeds accelerated aged for 15 d was higher than that for 12 d, increased by 2.6% (Figure 2a; Table 2).

Effects of Accelerated ageing on Germination Percentage and Germination Rate of Seeds

The final germination percentage of seeds and fresh weight of seedling produced by germinating seeds decreased with accelerated ageing, and decreased to zero until 15 d of accelerated ageing (Figure 2b; Table 2); the accelerated ageing time when final seed germination was decreased to 50% (GA_{50}) was about 10.7 d. Seed vigor, as measured by fresh weight of seedling produced by germinating seeds at 5 d of imbibition (Figure 2b; Table 2) and germination rate of seeds (Figure 2c), also decreased with increasing accelerated ageing time. The time required for 50% germination of seeds accelerated aged for 0, 3, 6, 9 d at 30°C were about 20, 20, 29, and 37 h, respectively, final germination percentage of seeds accelerated aged for 12 d were only 32.7%, and for 15 d, 0 (Figure 2c).

Changes in Superoxide Radical and Hydrogen Peroxide

$\cdot O_2^-$ production rate of seeds increased with accelerated ageing time, peaked at the 9 d of accelerated ageing, and then decreased; its production rate of seeds accelerated aged 15 d, however, was higher than that of non-aged seeds (Figure 3a). H_2O_2 content of seeds gradually increased with accelerated ageing; and compared to non-aged seeds, H_2O_2 content of seeds accelerated aged for 15 d increased by 25.6% (Figure 3a).

Activities of SOD, APX and CAT

SOD activities of seeds increased gradually during accelerated ageing up to 12 d, and then decreased; changes in APX activities were similar to those of SOD, but peaked at 6 d of accelerated ageing, and then decreased (Figure 3b).

CAT activity of seeds decreased significantly with accelerated ageing, decreased by 92% by the 15 d of accelerated ageing at which final germination percentage of seeds became zero, compared with non-aged axes (Figure 3b).

MDA Content

MDA content of seeds increased gradually with accelerated ageing until 12 d, and then decreased slightly. MDA content increased by 21% and 16%, respectively by the 12 d and 15 d of accelerated ageing, compared to non-aged seeds (Figure 3c).

Discussion

Temperature affects both the capacity for germination and the rate of germination of seed [5]. The final germination percentage of guar seeds and the time required for 50% of seed germination at 30, 35, 40°C was about 20 h, but fresh weight of seedling produced by germinating seeds was the largest at 30°C, and increased by 27.7% and 4.2% at 30°C than at 25°C and 35°C, respectively (Figure 1a). Therefore, it seems important to include final germination percentage

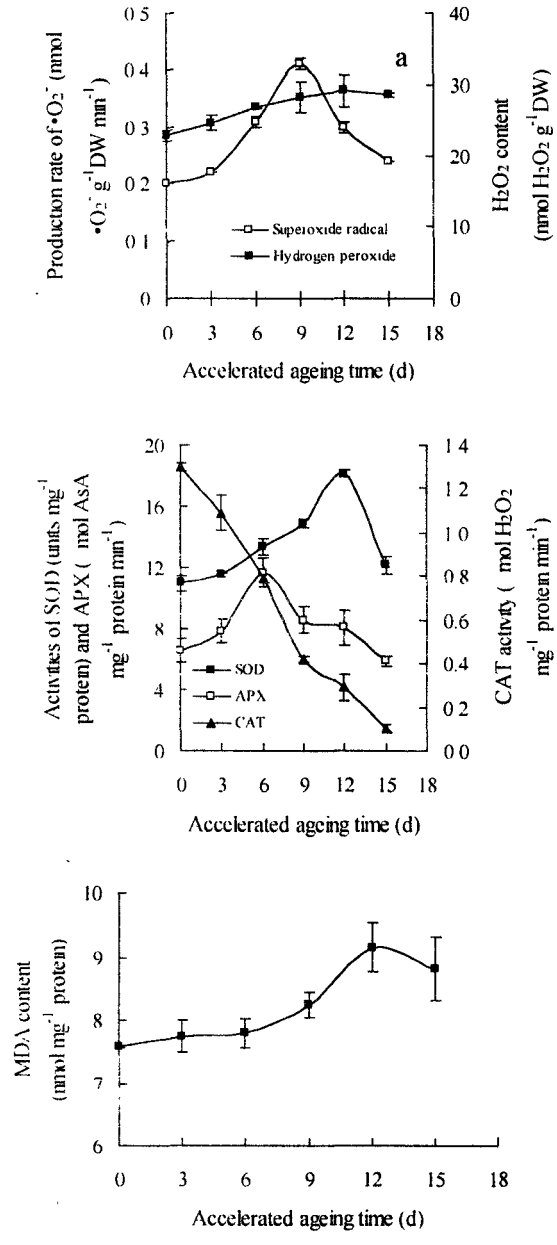

Figure 3. Changes in production rate of $\cdot O_2^-$, H_2O_2 content (a), activities of SOD, APX and CAT (b), and MDA content (c) during accelerated ageing of Cyamopsis tetragonoloba seeds. Accelerated ageing of seed, determination of production rate of $\cdot O_2^-$, H_2O_2 and MDA content, and assay of SOD, APX and CAT activities were carried out as described in Materials and Methods. All values are means ± SD of three replicates.

of seeds, fresh weight produced by germinating seeds and germination rate when discussing effect of temperature on seed germination.

The fact that seeds are seriously infected by storage fungi during accelerated ageing is usually encountered. The major deleterious effects of storage fungi are to (1) decrease viability, (2) cause discoloration, (3) produce mycotoxins, (4) cause heat production, and (5) develop mustiness and caking [5]. Therefore, to decrease or avoid infection by storage fungi is very important for studying physiological and biochemical changes of seeds during accelerated ageing. In an environment of 100% RH and 40°C, 1% hypochlorite, instead of water, could significantly avoid infection produced by storage fungi; infection percentage of guar seeds above 1% hypochlorite was decreased by 97.6% than above water (data not shown).

Under accelerated ageing (40°C, 100% RH), gradual increase in water content of seeds (Figure 2a) was caused by ambient 100% RH. Leakage rate of electrolyte of seeds rapidly increased at 3 d of accelerated ageing, and then slowly decreased up to 12 d, and finally increased slightly (Figure 1a; Table 2). Because water potential of ambient environment (100% RH) was much higher than that of dry seeds, the influx of water into the cells of dry seeds at the initial stage of accelerated ageing will result in temporary structural perturbations, particularly to membranes, which lead to an immediate and rapid leakage of solute and low molecular weight metabolites into the surrounding imbibition solution. This is a consequence of the transition of the membrane phospholipid components from the gel phase formed during maturation drying to the normal, hydrated liquid-crystalline state. With an increasing accelerated ageing, water content of seeds increased, the membranes returned to their more stable configuration, at which time solute leakage was decreased. Leakage rate of seeds accelerated aged for 15 d was larger than that of seeds for 12 d, showed that structure and function of membranes were re-destroyed with further accelerated ageing.

The final germination percentage (Figure 2b; Table 2) and germination rate (Figure 2c) of seeds, and fresh weight of seedling produced by germinating seeds decreased with increasing accelerated ageing, as found for *Arachis hypogaea* seeds by Song *et al.* [21], for wheat seeds by Guy and Black [8] and for *Beta vulgaris* seeds by Song *et al.* [22]. The symptoms observed during accelerated ageing can be used to characterize the degree of ageing, which varies in the opposite direction as storability. Stability against accelerated ageing has subsequently been recognized as a useful vigor test for some species [18]. The physiological and biochemical changes during rapid deterioration of seeds have been increasingly used as indices of ageing [18].

$\cdot O_2^-$ production rate of guar seeds increased with accelerated ageing time, peaked at the 9 d of accelerated ageing, and then decreased. H_2O_2 content of seeds increased also with accelerated ageing (Figure 3a). The production of ROS, such as $\cdot O_2^-$, and H_2O_2, is an unavoidable consequence of aerobic metabolism. In plant cell the mitochondrial electron transport chain is a major site of ROS production [14]. Song *et al.* [22] showed that activities and latencies of cytochrome c oxidase (EC 1.3.9.1) and malate dehydrogenase (EC 1.1.1.37) considerably decreased with accelerated ageing of *B. vulgaris* seeds.

Activities of SOD and APX of seeds increased gradually during early stage of accelerated ageing, and then decreased; CAT activity of seeds, however, decreased significantly with accelerated ageing (Figure 3b). MDA is one of the main products of lipid peroxidation. Compared with non-aged seeds, MDA content of seeds accelerated aged for 12 and 15 d

increased by 21% and 16%, respectively (Figure 3c). These results were in accordance with the findings for *A. hypogaea* seeds by Song *et al.* [21] and Sung and Jeng [24], for *Helianthus annuus* seeds by Bailly *et al.* [2], who demonstrated that loss of seed viability was associated with a decrease in SOD, CAT and APX, and that accelerated ageing could induce accumulation of MDA.

Based on experimental results mentioned above, it was considered that when seeds were subjected to accelerated ageing (40°C, 100% RH), production rate of $\cdot O_2^-$ and H_2O_2 content of seeds increased, the activities of SOD, APX and CAT decreased, lipid peroxidation increased, finally seed viability was gradually lost by these events. McDonald [13] considered that production of ROS which caused lipid peroxidation may be a principal cause of seed deterioration.

Acknowledgements

We are grateful to the Knowledge Innovation Project of the Chinese Academy of Sciences (KSCX2-SW-117), Hundreds Talent Program of the Chinese Academy of Sciences, and Natural Science Foundation of Yunnan (2003C0068M) of China for supporting.

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13 Enhanced Stress Tolerance in Plants through Genetic Engineering of Manganese Superoxide Dismutase

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Introduction

Environmental stress can affect physiological processes from seed germination to plant growth and development [1]. Plants have evolved different strategies to deal with environmental stress. For example, some of the angiosperm and gymnosperm species accumulate compatible molecules include betaines, sulfonium compounds, sugars, polyols and amino acids. Stress tolerance is developmentally regulated, stage-specific phenomena because tolerance at one stage of plant development is not necessarily correlated with tolerance at other stages [2,3]. Genetic engineering based on gene transfer technology provides opportunities to improve plant stress tolerance in the fields of agriculture, forestry, horticulture and environmental biotechnology. Transgenic plants expressing betaine aldehyde dehydrogenase (BADH) [4], which catalyses the last step of glycine-betaine synthesis located in peroxisomes in rice and barley [5], improved stress tolerance. Transgenic plants expressing a choline oxidase gene enhanced tolerance to salt stress [6,7]. In *Arabidopsis thaliana*, overexpression of a vacuolar Na^+/H^+ resulted in enhanced tolerance to salt [8]. Overexpression of two *Arabidopsis* ERF/AP2 genes, *CBF1/DREBP1B* and *DREBP1A*, resulted in enhanced tolerance to drought, salt, and freezing [9]. Overexpression of a putative R2R3-type MYB transcription factor in *Arabidopsis* increased low temperature and salt stress, and increased expression of ABA biosynthesis genes during stress [10].

Oxidative stress is one amongst the wide range of environmental stresses that influence the growth and yield of crop plant. Oxidative stress results in enhanced production of activated oxygen species (AOS) that are highly toxic to living cells [11-13]. Manganese superoxide dismutase (MnSOD E.C. 1.15.1.1.) is a nuclear-encoded protein that scavenges superoxide radicals in the mitochondrial matrix. MnSOD catalyzes the first step in the scavenging system of active oxygen by the disproportionation of superoxide anion radicals to hydrogen pe oxide

and oxygen [12-14]. MnSOD is synthesized in the cytoplasm as a preprotein and is subsequently translocated to the mitochondrial matrix with corresponding cleavage of an NH_2-terminal leader sequence. SOD has multiple isoforms, which are classified by their metal cofactors: copper/zinc (Cu/Zn), manganese (Mn), and iron (Fe) forms [11,15]. Mn-SOD and Fe-SOD are structurally very similar, while copper/zinc superoxide dismutase (Cu/Zn-SOD) is not related [11,16]. In higher plants, Cu/Zn-SOD is mainly located in plastids and cytosol, and Mn-SOD is predominantly in the mitochondrial matrix. Cu/Zn-SOD and Mn-SOD are found among all plant species, but Fe-SOD, which is located in chloroplasts [11,17], has been characterized only in several dicotyledonous plant species [18]. Mitochondrial manganese superoxide dismutase (Mn-SOD) has been isolated and characterized from *Capsicum annuum* L. [12,13]. The Mn-SOD was purified from pearl millet by ammonium sulfate precipitation followed by column chromatography using DEAE-cellulose and Sephadex G-100 [11]. The isozyme has a molecular weight of 35 kDa. Electrophoresis revealed a single band of SOD activity corresponding to the purified enzyme [11]. The purified pearl millet SOD exhibited insensitivity to hydrogen peroxide and cyanide, which is typical of Mn-containing SOD. SOD was purified 73-fold from pearl millet [11].

Manganese superoxide dismutase (MnSOD) is the principal antioxidant enzyme of mitochondria. Because mitochondria consume over 90% of the oxygen used by cells, they are especially vulnerable to oxidative stress. The superoxide radical is one of the reactive oxygen species produced in mitochondria during ATP synthesis. MnSOD catalyzes the conversion of superoxide radicals to hydrogen peroxide, which can be reduced to water by other antioxidant enzymes. Recently, two cDNA clones encoding mitochondrial manganese superoxide dismutases (MnSODs) from peach (*Prunus persica* [L.] Batsch) were identified, which show homologies to several plant MnSODs [19]. The amino acid sequence predicted from one full-length clone (MnSOD1) showed the highest homology to an MnSOD from *Nicotiana plumbaginifolia* (94%) and included a 24-amino acid transit peptide typical of those used to target proteins to the mitochondria [19]. A second, partial clone (MnSOD2) showed divergence from MnSOD1 in the 3' untranslated region [19]. It could therefore derive from a second gene or from an allele of MnSOD1. Southern hybridization analysis suggests the existence of two MnSOD genes in peach [19]. SOD isoenzyme profiles, MnSOD1 expression and protein levels were studied in aerial vegetative tissues derived from plants of different ages and in adult plants during the seasonal cycle [19]. Levels of MnSODs were lower in leaves derived from apical shoots of adult plants than in leaves derived from seedlings, basal shoots or *in vitro* propagated juvenile plants, which are considered as juvenile-like structures [19]. The MnSOD1 transcript and protein followed the same pattern. The results suggest that the steady-state levels of MnSOD1 mRNA in leaves vary with both the ontogenetic stage and the growth rate of the tissues examined [19]. We currently work on gene transfer of an MnSOD gene isolated from *Tamarix androssowii* into pine species. In this study, we focus on the following functions of MnSOD including: (1) enhances tolerance of freezing stress; (2) protect transgenic plants against ozone damage; (3) reduce oxidative damage; (4) confer resistance to oxidative agents and the fungus; (5) increase drought tolerance of transgenic plants; and (6) reduce cellular-damage mediated by oxygen radicals in transgenic plants.

MnSOD Enhances Tolerance of Freezing Stress

Plants use a wide array of proteins to protect themselves against low temperature and freezing conditions [20-22]. The identification of these freezing tolerance associated proteins and the elucidation of their cryoprotective functions will have important applications in several fields. Genes encoding structural proteins, osmolyte producing enzymes, oxidative stress scavenging enzymes, lipid desaturases and gene regulators have been used to produce transgenic plants [20,23,24]. These studies have revealed the potential capacity of different genes to protect against temperature related stresses. In some cases, transgenic plants with significant cold tolerance have been produced [20]. Furthermore, the biochemical characterization of the cold induced antifreeze proteins and dehydrins reveals many applications in the food and the medical industries [20]. These proteins are being considered as food additives to improve the quality and shelf-life of frozen foods, as cryoprotective agents for organ and cell cryopreservation, and as chemical adjuvants in cancer cryosurgery [20].

Activated oxygen or oxygen free radicals have been implicated in a number of physiological disorders in plants including freezing injury [25-27]. Superoxide dismutase (SOD) catalyzes the dismutation of superoxide into O_2 and H_2O_2 and thereby reduces the titer of activated oxygen molecules in the cell [25]. To further examine the relationship between oxidative and freezing stresses, the expression of SOD was modified in transgenic alfalfa (*Medicago sativa* L.) [25]. The Mn-SOD cDNA from *Nicotiana plumbaginifolia* under the control of the cauliflower mosaic virus 35S promoter was introduced into alfalfa using *Agrobacterium tumefaciens*-mediated transformation. Two plasmid vectors, pMitSOD and pChlSOD, contained a chimeric Mn-SOD construct with a transit peptide for targeting to the mitochondria or one for targeting to the chloroplast, respectively [25]. The putatively transgenic plants were selected for resistance to kanamycin and screened for neomycin phosphotransferase activity and the presence of an additional Mn-SOD isozyme. Detailed analysis of a set of four selected transformants indicated that some had enhanced SOD activity, increased tolerance to the diphenyl ether herbicide, acifluorfen, and increased regrowth after freezing stress [25]. The F1 progeny of one line, RA3-ChlSOD-30, were analyzed by SOD isozyme activity, by polymerase chain reaction for the Mn-SOD gene, and by polymerase chain reaction for the neo gene. RA3-ChlSOD-30 had three sites of insertion of pChlSOD, but only one gave a functional Mn-SOD isozyme; the other two were apparently partial insertions [25]. The progeny with a functional Mn-SOD transgene had more rapid regrowth following freezing stress than those progeny lacking the functional Mn-SOD transgene, suggesting that Mn-SOD serves a protective role by minimizing oxygen free radical production after freezing stress [25].

Superoxide dismutase (SOD) gene expression was investigated to elucidate its role in drought and freezing tolerance in spring and winter wheat (*Triticum aestivum*) [28]. cDNAs encoding chloroplastic Cu/ZnSODs and mitochondrial MnSODs were isolated from wheat. MnSOD and Cu/ZnSOD genes were mapped to the long arms of the homologous group-2 and -7 chromosomes, respectively [28]. Northern blots indicated that MnSOD genes were drought inducible and decreased after rehydration. In contrast, Cu/ZnSOD mRNA was not drought inducible but increased after rehydration. In both spring and winter wheat seedlings exposed to 2°C. MnSOD transcripts attained maximum levels between 7 and 49 d [28]. Transcripts of Cu/ZnSOD mRNA were detected sooner in winter than in spring wheat; however, they disappeared

after 21 d of acclimation. Transcripts of both classes of SOD genes increased during natural acclimation in both spring and winter types [28]. Exposure of fully hardened plants to three nonlethal freeze-thaw cycles resulted in Cu/Zn mRNA accumulation; however, MnSOD mRNA levels declined in spring wheat but remained unchanged in winter wheat [28]. The results of the dehydration and freeze-thaw-cycle experiments suggest that winter wheat has evolved a more effective stress-repair mechanism than spring wheat [28].

Transcript accumulation and protein activation of superoxide dismutase (SOD) isoenzymes during cold acclimation in potato genotypes of varying degrees of freezing tolerance had been studied [29]. Increased SOD activity and improved freezing tolerance were observed in all genotypes after cold acclimation for 2 days. In freezing-tolerant *Solanum commersonii* Dun, CuZnSOD isoenzyme activity increased more, compared to freezing sensitive *S. tuberosum* [29]. In potato hybrids (*S. commersonii* × *S. tuberosum* SPV11), there was no correlation between SOD activity and freezing tolerance [29]. Freezing tolerance continued to improve up to 7 days during cold acclimation. However, mitochondrial MnSOD transcript was not always detected. Seppanen and Fagerstedt [29] suggested that MnSOD transcript accumulation was probably an indicator of mitochondrial activity during cold acclimation.

MnSOD Protects Transgenic Plants against Ozone Damage

To evaluate the feasibility of using engineered antioxidant enzymes as an approach to improve the tolerance of plants to ambient stress, we have constructed transgenic tobacco plants that overproduce superoxide dismutase (SOD), an enzyme that converts superoxide radicals into hydrogen peroxide and oxygen, and is believed to play a crucial role in antioxidant defense [30]. Vancamp *et al.* [30] have targeted the MnSOD from *Nicotiana plumbaginifolia* either to the chloroplasts or to the mitochondria, and evaluated the ozone tolerance of transgenic and control plants. Enhanced SOD activity in the mitochondria had only a minor effect on ozone tolerance. However overproduction of SOD in the chloroplasts resulted in a 3-4 fold reduction of visible ozone injury [30].

Chloroplast-targeted overexpression of a Fe superoxide dismutase (SOD) from *Arabidopsis thaliana* resulted in substantially increased foliar SOD activities [31]. Ascorbate peroxidase, glutathione reductase, and monodehydroascorbate reductase activities were similar in the leaves from all of the lines, but dehydroascorbate reductase activity was increased in the leaves of the FeSOD transformants relative to untransformed controls [31]. Foliar H_2O_2, ascorbate, and glutathione contents were comparable in all lines of plants. Irradiance-dependent changes in net CO_2 assimilation and chlorophyll a fluorescence quenching parameters were similar in all lines both in air (21% O_2) and at low (1%) O_2. CO_2-response curves for photosynthesis showed similar net CO_2-exchange characteristics in all lines [31]. In contrast, values of photochemical quenching declined in leaves from untransformed controls at intercellular CO_2 (Ci) values below 200 $\mu L\ L^{-1}$ but remained constant with decreasing Ci in leaves of FeSOD transformants [31]. When the O_2 concentration was decreased from 21 to 1%, the effect of FeSOD overexpression on photochemical quenching at limiting Ci was abolished. At high light (1000 $\mu mol\ m^{-2}\ s^{-1}$) a progressive decrease in the ratio of variable (F_v) to maximal (F_m) fluorescence was observed with decreasing temperature. At 6°C the high-light-induced decrease in the F_v/F_m ratio was partially prevented by low O_2 but values were comparable in all lines

[31]. Methyl viologen caused decreased F_v/F_m ratios, but this was less marked in the FeSOD transformants than in the untransformed controls. These observations suggest that the rate of superoxide dismutation limits flux through the Mehler-peroxidase cycle in certain conditions [31].

MnSOD Reduces Oxidative Damage

Superoxide dismutases (SODs) are metal-containing enzymes that catalyze the dismutation of superoxide radicals to oxygen and hydrogen peroxide [15]. The enzyme has been found in all aerobic organisms examined where it plays a major role in the defense against toxic-reduced oxygen species, which are generated as byproducts of many biological oxidations [15,22-24]. The generation of oxygen radicals can be further exacerbated during environmental adversity and consequently SOD has been proposed to be important for plant stress tolerance [15]. In plants, three forms of the enzyme exist, as classified by their active site metal ion: copper/zinc, manganese, and iron forms. The distribution of these enzymes has been studied both at the subcellular level and at the phylogenic level [15]. It is only in plants that all three different types of SOD coexist. Their occurrence in the different subcellular compartments of plant cells allows a study of their molecular evolution and the possibility of understanding why three functionally equivalent but structurally different types of SOD have been maintained [15]. Several cDNA sequences that encode the different SODs have recently become available, and the use of molecular techniques have greatly increased our knowledge about this enzyme system and about oxidative stress in plants in general, such that now is an appropriate time to review our current knowledge [15,22-24].

Plants are confronted on a regular basis with a range of environmental stresses [32-34]. These include abiotic insults caused by, for example, extreme temperatures, altered water status or nutrients, and biotic stresses generated by a plethora of plant pathogens. Many studies have shown that the cellular responses to these environmental challenges are rather similar, which might be why plants resistant to one stress are sometimes cross-tolerant to others [32,34]. To understand this phenomenon and to be able to take full advantage of it in agriculture, we must determine whether the individual biochemical pathways that make up the responses to each external stimulus are activated by unique, overlapping or redundant signaling systems [32]. Bowler and Fluhr [32] discuss the potential role of signaling molecules, such as calcium and activated oxygen species, in underlying cross-tolerance [32,34].

MnSOD Confers Resistance to Oxidative Agents and the Fungus

Sugarbeets carrying superoxide dismutase transgenes were developed in order to investigate the possibility of enhancing their resistance to oxidative stress [22-24,35]. Binary T-DNA vectors carrying the chloroplastic and cytosolic superoxide dismutase genes from tomato were used for *Agrobacterium*-mediated transformation of sugarbeet petioles [35]. The transgenic plants were subjected to treatments known to cause oxidative stress, such as the herbicide methyl viologen and a natural photosensitizer toxin produced by the fungus *Cercospora beticola*, namely cercosporin [35]. The transgenic plants exhibited increased tolerance to methyl viologen, to pure cercosporin, as well as to leaf infection with the fungus *C. beticola* [35].

Reactive oxygen species (ROS), such as singlet oxygen, superoxide radical, hydrogen

peroxide, hydroxyl anion and free hydroxyl radical are products of the normal cellular metabolism and are known to cause oxidative damage to living tissues by oxidizing cellular components such as lipids, proteins, carbohydrates and nucleic acids [22-24,35]. Elevated levels of ROS can also arise as a result of adverse environmental conditions and chemical agents, such as heat and drought, intense light, excessively low temperatures, herbicides and parasitic pathogens [35,36]. An important pathogen affecting sugarbeet (*Beta vulgaris* L.) is the fungus *Cercospora beticola*, which attacks the leaves and causes leaf damage and reduced sugar content [35]. Protection against oxidative stress is complex and includes both enzymatic and non-enzymatic components [35]. One of the key enzymatic systems in this defense are superoxide dismutases (SODs), which scavenge superoxide radicals and convert them into hydrogen peroxide [37]. Physiological correlations between elevated SOD activity and stress tolerance have been reported [22-24,37], suggesting that the upregulation of SOD levels may enhance the stress-defense potential of plants. Tertivanidis *et al.* [35] show for the first time that the SOD-transformed plants exhibited increased resistance not only to oxidative stress caused by methyl viologen (MV) but also to the fungal toxin cercosporin, as well as to leaf infection with the fungus *C. beticola* [35].

MnSOD Increases Drought Tolerance of Transgenic Plants

Wang *et al.* [38] investigated the role that manganese superoxide dismutase (MnSOD), an important antioxidant enzyme, may play in the drought tolerance of rice. MnSOD from pea (*Pisum sativum*) under the control of an oxidative stress-inducible SWPA2 promoter was introduced into chloroplasts of rice (*Oryza sativa*) by *Agrobacterium*-mediated transformation to develop drought-tolerant rice plants [38]. Functional expression of the pea MnSOD in transgenic rice plants (T1) was revealed under drought stress induced by polyethylene glycol (PEG) 6000 [38]. After PEG treatment the transgenic leaf slices showed reduced electrolyte leakage compared to wild type (WT) leaf slices, whether they were exposed to methyl viologen (MV) or not, suggesting that transgenic plants were more resistant to MV- or PEG-induced oxidative stress [38]. Transgenic plants also exhibited less injury, measured by net photosynthetic rate, when treated with PEG. Wang *et al.* [38] suggest that SOD is a critical component of the ROS scavenging system in plant chloroplasts and that the expression of MnSOD can improve drought tolerance in rice [22-24,38].

Injury caused by ROS, known as oxidative stress, is one of the major damaging factors in plants exposed to environmental stresses such as drought [39], desiccation [40] and high light intensity [41]. Chloroplasts are particularly susceptible to ROS because of the relatively high concentration of oxygen that reacts with electrons which escape from the photosynthetic electron transfer system [42]. ROS can affect a variety of biological macromolecules, lead to severe cellular damage, inhibit photosynthesis and hence reduce the yield of crops. Plants have evolved a wide range of enzymatic and non-enzymatic mechanisms to scavenge ROS. The level of enhancement of SOD activity under stress conditions resulting from introducing SOD genes into plants may result in more efficient elimination of ROS. Several transgenic plants with extragenetic SODs resulting in over-expression primarily in the chloroplasts have been reported. SOD transformants varied in their protective response to oxidative stresses, induced by environmental stresses such as chilling, ozone, water deficit and salt [42]. The observation that

plants under drought stress generate reactive oxygen species [39] and the fact that transgenic alfalfa expressing MnSOD has reduced injury from water deficit [43] prompted us to study whether rice expressing foreign SOD under the control of a stress inducible promoter would improve drought tolerance. In this study, we developed transgenic rice plants that express pea MnSOD in chloroplasts under the control of a stress inducible SWPA2 promoter [44], and compared tolerance to oxidative stress and photosynthesis rates between wild type and T1 transgenic plants under drought stress mediated by PEG.

MnSOD Reduced Cellular Damage Mediated by Oxygen Radicals

In plants, environmental adversity often leads to the formation of highly reactive oxygen radicals. Since resistance to such conditions may be correlated with the activity of enzymes involved in oxygen detoxification, Bowler *et al.* [45] have generated transgenic tobacco plants which express elevated levels of manganese superoxide dismutase (MnSOD) within their chloroplasts or mitochondria [45]. Leaf discs of these plants have been analyzed in conditions in which oxidative stress was generated preferentially within one or the other organelle [45]. It was found that high level overproduction of MnSOD in the corresponding subcellular location could significantly reduce the amount of cellular damage, which would normally occur. In contrast, small increases in MnSOD activity were deleterious under some conditions [22-24,45]. A generally applicable model correlating the consequences of SOD with the magnitude of its expression was presented by Bowler *et al.* [45].

Superoxide dismutases (SODs) are metalloproteins that catalyze the dismutation of superoxide radicals to hydrogen peroxide and oxygen [46]. The enzyme is ubiquitous in aerobic organisms where it plays a major role in defense against oxygen radical-mediated toxicity [46]. In plants, environmental adversity often leads to the increased generation of reduced oxygen species and consequently, SOD has been proposed to be important in plant stress tolerance. Here we describe the isolation of a cDNA clone encoding a cytosolic copper/zinc SOD from *Nicotiana plumbaginifolia* [46]. Using this, together with previously isolated cDNAs encoding the mitochondrial manganese SOD and the chloroplastic iron SOD as probes in RNA gel blot analyses, we have studied SOD transcript abundance during different stress conditions: in response to light, during photoinhibitory conditions (light combined with high or low temperatures), and in response to a xenobiotic stress imposed by the herbicide paraquat. Evidence is presented that iron SOD mRNA abundance increases whenever there is a chloroplast-localized oxidative stress, similar to the previous finding that manganese SOD responds to mitochondria-localized events [46]. The diverse effects of the different stress conditions on SOD mRNA abundance thus might provide an insight into the way that each treatment affects the different subcellular compartments [22,23,46].

Functional Genomics of MnSOD

Manganese superoxide dismutase (MnSOD) plays an important role in: (1) enhances tolerance of freezing stress; (2) protect transgenic plants against ozone damage; (3) reduce oxidative damage; (4) confer resistance to oxidative agents and the fungus; (5) increase drought tolerance of transgenic plants; and (6) reduce cellular-damage mediated by oxygen radicals in transgenic plants. To increase our understanding of MnSOD function in plant stress tolerance, it is needed

to investigate expression profile of MnSOD in a genome-scale. Genome-wide study of MnSOD will speed the application of MnSOD transgenic plants in agricultural and environmental industries. In pea (*Pisum sativum* L.) leaves, expression analysis of manganese superoxide dismutase (Mn-SOD) cDNA demonstrated that MnSOD is mainly localized in mitochondria as well as in peroxisomes [47]. Northern blots hybridized with a cDNA encoding mitochondrial Mn-SOD from pea leaves and a mitochondrial Mn-SOD transit peptide-specific probe showed increased Mn-SOD transcript levels during leaf senescence [47]. Western blot assays of crude extracts with the antibodies to pea mitochondrial Mn-SOD showed that the levels of total Mn-SOD protein gradually increased with leaf senescence [47]. It is reported that mitochondrial and peroxisomal Mn-SOD expression is regulated differently. The expression of mitochondrial Mn-SOD is induced during the senescence of pea leaves, whereas peroxisomal Mn-SOD could be post-translationally activated [47]. In pearl millet (*Pennisetum typhoides*), Mn-SOD protein was purified from seedlings by ammonium sulfate precipitation followed by column chromatography using DEAE-cellulose and Sephadex G-100 [11]. Electrophoresis revealed a single band of SOD activity corresponding to the purified enzyme. The purified enzyme has a molecular weight of 35 kDa and has exhibited insensitivity to hydrogen peroxide and cyanide, which is typical of Mn-containing SOD [11]. The purified pearl millet SOD was stable over a pH range of 7.0-9.0 for 24 h and a temperature range of 20-35°C [11]. Expression profiles of MnSOD will be very valuable in functional genomics study of plant stress tolerance and genetic engineering of tree [48]. A conclusion of MnSOD gene isolated from different species has been included in Table 1 and functions of several important MnSOD genes were demonstrated

Table 1. Sequences producing significant alignments after blasting of *Triticum aestivum* manganese superoxide dismutase cDNA (U73172) through NCBI Blast

Species	*Locus of gene*	*Score (Bits)*	*E value*
1	*2*	*3*	*4*
Triticum aestivum	AF092524.1	1191	0.0
Zea mays	L19463.1	724	0.0
Oryza sativa	AK104160.1	714	0.0
Ipomoea batatas	L77078.1	131	4e-27
Cinnamomum camphora	AF084831.1	123	1e-24
Zantedeschia aethiopica	AF094832.1	121	4e-24
Barbula unguiculata	AB028460.1	119	2e-23
Raphanus sativus	AF061333.1	107	6e-20
Musa acuminata	AF510071.1	93.7	9e-16
Arabidopsis thaliana MSD1	NM001035593.1	91.7	4e-15
Sorghum bicolor	AY504100.1	89.7	1e-14
Raphanus sativus	AF263920.1	85.7	2e-13
Capsicum annuum	AF036936.2	83.8	9e-13
Digitalis lanata	AJ278863.1	79.8	1e-11
Ipomoea batatas	L36676.1	79.8	1e-11
Camellia sinensis	AY641734.2	75.8	2e-10

1	*2*	*3*	*4*
Tamarix androssowii	AY573576.2	75.8	2e-10
Glycine max	AJ440726.1	75.8	2e-10
Prunus persica mitochondria	AJ238316.2	75.8	2e-10
Citrus maxima voucher Y.Liu 1046	DQ193969.1	73.8	9e-10
Citrullus lanatus	AY542525.1	73.8	9e-10
Callinectes sapidus mitochondria	AF264029.1	73.8	9e-10
Charybdis feriatus	AF019411.1	73.8	9e-10
Zea mays clone EL01N0312A05.c	BT017268.1	71.9	3e-09
Avicennia marina	AY137205.1	71.9	3e-09
Citrus limon voucher Y.Liu 1103	DQ193968.1	69.9	1e-08
Citrus maxima voucher Y.Liu 1015	DQ193967.1	69.9	1e-08
Rhodotorula glutinis	AF434197.1	69.9	1e-08
Nicotiana tabacum	X14482.1	67.9	5e-08
Biomphalaria glabrata strain BS90	AY500814.1	67.9	5e-08
Biomphalaria glabrata	AY500813.1	67.9	5e-08
Nicotiana tabacum	AB093097.1	67.9	5e-08
Citrus maxima voucher Y.Liu 1037	DQ193966.1	65.9	2e-07
Citrus maxima voucher Y.Liu 1011	DQ193965.1	65.9	2e-07
Poncirus trifoliata	DQ193964.1	65.9	2e-07
Penaeus monodon	AY726542.1	65.9	2e-07
Ustilago maydis	XM754139.1	65.9	2e-07
Danio rerio	AY195857.1	65.9	2e-07
Gossypium hirsutum	AF061514.1	63.9	8e-07
Mycobacterium lepraemurium	D13288.1	63.9	8e-07
Ixodes pacificus	AY674270.1	61.9	3e-06
Lycopersicon esculentum	BT013288.1	61.9	3e-06
Hevea brasiliensis	AJ289158.1	61.9	3e-06

in Table 2. In our laboratory, we observed that overexpression of a *Tamarix androssowii* MnSOD conferred freezing tolerance in transgenic *Pinus strobus* (Figure 1). An overview of signal transduction pathways involved in MnSOD expression in plant and animal species [49] is included in Figure 2. All of these progresses can be very useful to genetic engineering of MnSOD in plant stress biotechnology.

Acknowledgements

The authors are grateful to Dr. Y.C. Wang for the MnSOD cDNA, and to undergraduate research assistants Karina Hesterberg, Ruba Sarsour, Adaeze Okoye, Hubert Chia and Zalak Daftary, for their work in isolating mature embryos from seeds for callus induction. This work was supported by the East Carolina Christmas Tree Program.

Table 2. Functional study of the manganese superoxide dismutase (MnSOD) gene in different plant species

Species	*Name of gene*	*Function*	*References*
Pea (*Pisum sativum* L.)	Manganese superoxide dismutase (Mn-SOD)	Mitochondrial and peroxisomal Mn-SOD expression is regulated differently. The expression of mitochondrial Mn-SOD is induced during the senescence of pea leaves, whereas peroxisomal Mn-SOD could be post-translationally activated. The described results showing decreased mitochondrial Mn-SOD activity and increased peroxisomal Mn-SOD activity may be reflective of post-translational events regulating enzymatic activity during leaf senescence.	[47]
Pearl millet [*Pennisetum glaucum* (L.)]	Manganese superoxide dismutase (Mn-SOD)	The downy mildew resistant pearl millet genotype showed a distinctly high SOD activity on inoculation with *S. graminicola*.	[11]
Rice (*Oryza sativa*)	Manganese superoxide dismutase (Mn-SOD)	The data suggest that SOD is a critical component of the ROS scavenging system in plant chloroplasts and that the expression of MnSOD can improve drought tolerance in rice.	[38]
Peach (*Prunus persica* [L.] Batsch)	Manganese superoxide dismutase (Mn-SOD)	The results suggest that the steady-state levels of *MnSOD1* mRNA in leaves vary with both the ontogenetic stage and the growth rate of the tissues examined.	[19]
Potato [*Solanum. tuberosum* (SPV11)]	Manganese superoxide dismutase (Mn-SOD)	Results suggest that although SOD activity may contribute to the freezing tolerance and acclimation capacity, traits other than SOD activity were more important for the expression of freezing tolerance in potato hybrids.	[29]
Wheat (*Triticum aestivum*)	Manganese superoxide dismutase (Mn-SOD) and Cu/ZnSOD	Cu/ZnSOD mRNA was not drought inducible. MnSOD transcripts attained maximum levels between 7 and 49 d. Transcripts of Cu/ZnSOD mRNA were detected sooner in winter than in spring wheat. Transcripts of both classes of SOD genes increased during natural acclimation in both spring and winter types. Exposure of fully hardened plants to three nonlethal freeze-thaw cycles resulted in Cu/Zn mRNA accumulation. These results suggest that winter wheat has evolved a more effective stress-repair mechanism than spring wheat.	[28]

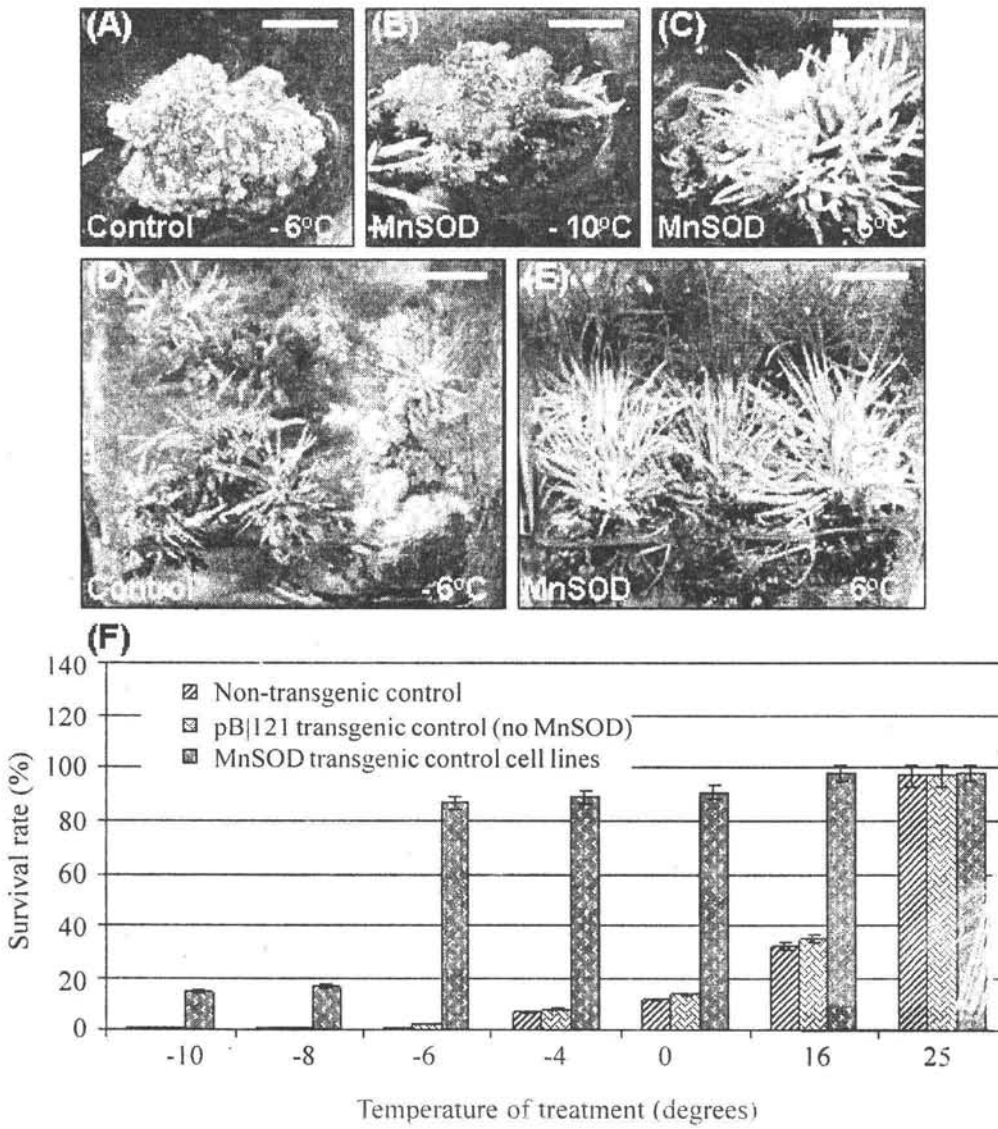

Figure 1. Freezing tolerance of transgenic pine calli induced from mature zygotic embryos, regenerated adventitious shoots and plantlets. Among different temperature conditions (-10, -8, -6, -4, 0, 16 and 25°C) of treatment used in the study, MnSOD transgenic plants are tolerance to temperature higher than -6°C, but not -10°C (B, C, E and F). However, non-transgenic controls of calli, shoots and plantlets died under -6°C or reduced survival rate under 16°C condition (A, D, and F).

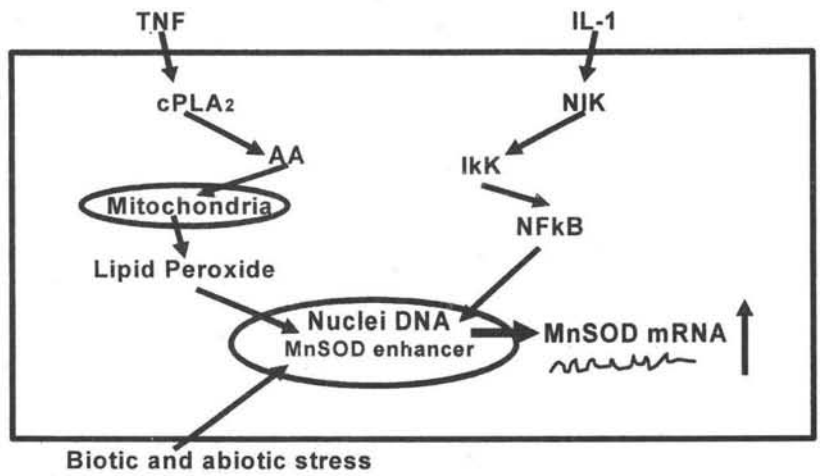

Figure 2. An overview of signal transduction pathways involved in MnSOD expression in plant and animal species, which are involved in the TNF-a signaling pathway and the IL-1 pathway (Rogers et al., 2001). Biotic and abiotic stress such as drought, stress and freezing induced, expression of MnSOD in plant species through an unidentified pathway. AA: arachidonic acid; NF-kB: the Ik subunit from nuclear factor kB.

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14 Genetic Transformation and Abiotic Stress Improvement in Transgenic Tomatoes Expressing Master Switch *Arabidopsis CBF*1 Gene

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Introduction

Tomatoes (*Lycopersicon esculentum* L. Miller) are nutritious, rich in vitamin A and C, one of the largest commercial vegetable crop, and covers more than 3.9 million-hectares worldwide with an annual production of 108 million metric tones in the year 2002 [1]. However, large proportion of crop yield is lost due to the detrimental effects of abiotic stress conditions including extremes in low temperatures, drought, salinity and pathogens (biotic stress). In many cases both abiotic and biotic factors together contribute to the severity of disease and yield losses. Attempts to improve the cold tolerance of tomato crop through conventional breeding programs have met with very limited success, due to the complexity of the trait; cold tolerance is complex genetically and physiologically and the success of breeding programs with the ultimate goal of improving crop productivity in tomato is limited by the lack of a clear understanding of the molecular basis of cold stress tolerance.

In order to survive and to continue progeny, irrespective of environmental conditions, plants evolved numerous physiological adaptations, which have been attributed to the function of various determinant genes and their products [2]. Sub-optimal growth conditions results in disruption of the coordinated action of biochemical pathways, which are otherwise instrumental in maintaining cellular homeostasis. Cold or non-freezing stress is an ever-present threat to crop yields especially in tropical and subtropical region and limits the growing season, whereas plants from temperate regions can survive freezing temperatures, a phenomenon known as cold acclimation [3]. It is significant that the maximum freezing tolerance of plants is not constitutive, but is induced in response to low temperatures, involves precise regulation of expression of transcription factors and effector genes collectively known as cold-regulated (COR) genes [4]. Today, significant progress has been made in identifying the regulators of cold-induced expression of COR genes and it is thought that their gene products may play

pivotal roles in drought and cold tolerance [5]. The main emphasis throughout the remainder of this chapter will be on genetic transformation, heterologous expression *CBF1* in transgenic tomato plants, abiotic stress tolerance and responsive gene(s).

Overview of *Arabidopsis* CBF Gene

A number of studies demonstrated that changes in gene expression occur in *Arabidopsis* in response to low temperature, such as KIN1, COR15a and COR6.6, and promoters regions of these genes included a 9 bp (TACCGACAT) DNA regulatory element called as dehydration-responsive element (DRE) [3,6]. The DRE elements contains a 5 bp core sequence of CCGAC, also known as C repeat (CRT), that plays an important role in regulation of gene expression in response to low temperature, water deficit and high salinity [6,7]. A family of DNA-binding proteins that bind to the DRE/CRT element and mediate transcription were isolated by the yeast one-hybrid method [8] and named DRE-binding proteins or (*DREB*s)/CRT-binding factors [9]. The first three of these DNA-binding proteins recognized as *CBF1, CBF2* and *CBF3* or *DREB1B*, *DREB1C* and *DREB1A*, respectively [9-12].

When plants are grown at normal conditions, the *CBF1*-3 genes are not expressed. However, transcripts of all three genes accumulate rapidly upon transferring plants to low temperature (within 15 min. at 4^{0}C) [13]. It indicated that CBF cold-responsive pathway is composed of two fundamental regulatory steps, such as rapid accumulation of CBF transcripts in response to low temperature and subsequent CBF transcriptional activators induce expression of genes containing CRT/DRE regulatory elements leads to an increase in freezing tolerance [9,13,14]. Recently, *CBF4/DREB2A*, additional AP2/ERF domain protein was reported [15], closely related to *CBF*1-3 and induced in response to drought and high salinity, but not low temperature. Accumulated evidences shown that when plants overexpressing the CBF gene are not only cold tolerant, but are also tolerant to other abiotic stress including drought, high salinity and other stressful conditions that lead to cellular dehydration [9,14,15]. Like *Arabidopsis*, tomato genome has three genes encoding CBF-like proteins that are present in tandem array and designated *LeCBF1*-3 [16]. The amino acid sequence of the three LeCBF proteins are 70-84% identical to each other and share 51-59% sequence identity to the sequence of *Arabidopsis CBF1*-3. Like *Arabidopsis CBF1*-3 genes, the *LeCBF1* gene in tomato is induced in response to low temperature, however, *LeCBF*2,3 are not responding, but are induced in response to mechanical agitation. Furthermore, experiments suggested that like *Arabidopsis CBF1*-3. *LeCBF*1-3 genes of tomato are not induced in response to drought or high salt stresses [16,17].

CBF Proteins and Cold Stress Tolerance

The dehydration-responsive element binding protein-1 (*DREB1*) also called *CBF* family, *DREB1A* (*CBF*3), *DREB1B* (*CBF*1), and *DREB1C* (*CBF*2) tandomly located on *Arabidopsis* chromosome 4 [11]. These genes are responsive to low temperature but not ABA (abscisic acid), according to deduced amino acid sequences all of the *DREB1* family encode transcriptional activators containing AP2 DNA binding domains [11,12] and can activate reporter genes containing CRT/DRE (C-repeat/ dehydration-responsive) elements in the promoter regions in yeast system [10,11]. This indicates that the *DREB1* family can bind to CRT/DRE elements, present in the promoters of *COR* (cold-regulated) genes, such as *KIN1*, *COR15a*, *COR47*, and

RD29A thus inducing these genes under low temperature and dehydration [9-14,18]. Moreover, overexpression of cDNA encoding *DREB1A* in transgenic *Arabidopsis* activated several stress tolerance genes and conferred enhanced tolerance to freezing-, salt- and drought-stresses [9,11,14].

The CBF family is highly conserved in *Arabidopsis*; more than 140 proteins that contain an AP2/ERF DNA-binding domain have been reported [19]. In addition, CBF genes have been identified in other species include the CBF signature sequences, such as *Brassica napus* [20], *Hordeum. vulgare* [21] *Triticum aestivum* [22], *L. esculentum* [16] *Oryza sativa* [23], *Secale cereale* [17], *Atriplex hortensis* [22], *Zea mays* [24], *Capsella bursa-pastoris* [25] and *Prunus avium* [26]. The homologous CBF proteins share the highest conservation in the AP2 domain. Short highly conserved polypeptide sequence (signature sequences), PKK/RPAGRxKFxETRHP and DSAWR, flank the AP2 domain and distinguish the CBF family from other AP2/EREBP domain containing proteins [17]. Furthermore, plant proteins only exclusively containing the AP2/ERF domain, and grouped *Arabidopsis* AP2/ERF proteins into five subfamilies according to amino acid sequence similarities [18]. The sequences within the AP2/ERF domain have been involved in determining the specificity of CBF/DREB1 binding to the CRT/DRE regulatory element. Henceforth, the *CBF*1-3/*DREB1A*-C' proteins belong to the DREB subfamily of AP2/ERF proteins with unique characteristic features that sets them apart from most of the other AP2/ERF proteins. *CBF*1/*DREB1B* a homolog of *DREB1A*, is a transcriptional activator that binds to the CRT/DRE element in the promoter region of COR genes that respond to both low temperature and water deficit [10,11]. The CBF overexpression in *Arabidopsis* results in the increased expression of many COR genes and an increase in freezing tolerance [11,13]. Additionally, expression of CBFs in heterologous systems demonstrated that CBF function is conserved in plants [17,22,26-29].

Crop Improvement in Tomato

Development of Gene Transformation Technology

In the last century, efforts through traditional plant breeding techniques have increased many agronomic aspects (biotic and abiotic) and nutritional values in tomato. However, the main drawback of traditional plant breeding is that it relies on the use of germplasm of the same or closely related species, which is sometimes a serious limiting factor. In addition, progress is time-consuming and relies on the extensive use of natural resources. The 21st century will likely pose a new set of challenges for growers and consumers, as an increased demand for tomato and its products will require novel production practices that sustain yield and quality while minimizing damage to the environment and the food chains vital to human health. Sustaining both supply and demand in the future will depend more heavily on the development and deployment of a range of new technologies, including biotechnology. The technology to produce transgenic plants will have an important and powerful impact on some of the immediate problems of vegetable crops, such as abiotic stresses and phytopathogens attack, and could reduce dependence on chemical pesticides and fungicides.

Plant transformation is an essential tool, both for the experimental investigation of gene function and for the improvement of crops either by enhancing existing traits or introducing new genes. It is now possible to introduce and express DNA stably in different plant species

irrespective of their origin, and many aspects of plant physiology and biochemistry that cannot be addressed easily by any other experimental means can be investigated by the analysis of gene function and regulation in transgenic plants. This offers an unprecedented opportunity to study the molecular basis of complex signal transduction pathways, development and reproduction. Introduction of DNA into plants was first attempted in the 1960s, due to lack of selectable markers and molecular tools to confirm transgene integration and expression made the outcome of such experiments unclear. A breakthrough came in the late 1970s with the elucidation of the mechanism of crown gall formation by *Agrobacterium tumefaciens* [30]. The discovery that virulent strains of *A tumefaciens* carried a large plasmid that conferred the ability to induce crown galls and that part of the plasmid (the T-DNA) was transferred to the plant genome of crown gall cells provided a natural gene transfer mechanism that could be exploited for plant transformation [31]. Tobacco plants carrying recombinant T-DNA sequences were first generated in 1981, although the foreign genes were driven by their own promoters and were not expressed in plant cells. The first transgenic tobacco plants expressing recombinant genes in integrated T-DNA sequences were reported in 1983 [32]. The technique of *Agrobacterium*-mediated transformation has been developed and refined since then to become a widely used technique for gene transfer into different crops. Because of commercial value, as a subject of research *in vitro* culture of tomatoes was initiated during early 1970's particularly on plant regeneration from a wide range of tissues and organs [33].

The earliest report of *Agrobacterium*-mediated transformation of tomato was nearly 20 years ago [34]. Other methods for introducing foreign DNA into tomato, including microprojection, particle bombardment, electroporation and polyethylene glycol (PEG)-mediated transformation of protoplasts have also been described [35-37]. However, none of these techniques has proven to be as popular as the *Agrobacterium*-mediated method, which is favored for its practicality, effectiveness and efficiency. In the past decades a mounted numbers of research reports on tomato transformation were witnessed particularly on the optimization of explant type and source, plant growth regulator for regeneration, *Agrobacterium* concentration, *Agrobacterium* virulent gene inducers and improvement in transformation efficiency to enable introduction of wide range of agronomically important genes (Table 1).

A Case Study of Heterologous Expression of *Arabidopsis* CBF1 Gene in Tomato

Many plants, including tomato, increase their ability to withstand low temperatures results largely from global changes in gene expression [3]. The changes in gene expression associated with clod acclimation resulting from the integration of multiple signal transduction pathways depending on the interaction of transcription factors with *cis-acting* elements and/or with other transcription factors required for gene expression [38]. CBFs, also known as DREBs, dehydration-responsive element binding proteins encode a small family of transcriptional activators containing an AP2 DNA-binding domain, which interacts specifically with the CRT/DRE. This element has been identified in 80% of genes assigned to the CBF regulon and CRT/DRE-like elements have been found in the promoters of both dicot and monocot genes, including *Brassica napus* [39], *Betula pendula* [40], *WCOR15* [41], *Hordeum vulgare* [42] and number of dehydrins [43] indicating that this pathway is conserved among distantly related species with varied cold tolerance.

Recent attempts to improve plant growth and performance by ectopic expression of stress responsive genes through genetic engineering have focused more on genetic model systems such as *Arabidopsis* and tobacco. Attractive attributes of these plants include a well established and reliable transformation system and quick growth. The beneficial effects observed in some of these transgenic plants could lead to interesting agronomic applications. To test the feasibility of this concept in crop plants, we introduced *Arabidopsis CBF1/DREB1B* cDNA into the tomato genome, and transgenic plants displayed enhanced tolerance to chilling-, salt-, oxidative- and water deficit stresses [27-29]. Our results implicated that *Arabidopsis DREB1B* can function effectively in transgenic tomato plants, and further suggesting that similar signal transduction pathway(s) pertaining to salt, chilling tolerance and water deficit resistance may exist in both *Arabidopsis* and tomato [9,10,13,14,18].

These observations are concordance with the stress inducible promoter from barley (ABRC1) driven *CBF1* transformed into tomato by *Agrobacterium*-mediated transformation. The biochemical basis for maintaining the cellular homeostasis and conferring tolerance to chilling, drought and salt stress has been depicted. The results demonstrate that the production of key ROS scavenging enzymes such as APX, SOD, glutathione reductase, peroxidase have a bearing in conferring tolerance in transgenic tomato plants under various stress conditions [29].

Transgenic CBF1 Tomato Plants are Tolerant to Various Abiotic Stresses

Cold Stress

Tomatoes are generally considered cold sensitive and do not cold acclimate unlike other plants, we showed that transgenic tomato plants constitutively expressing *Arabidopsis CBF1* can increase the degree of chilling tolerance than wild-types [27]. The photosynthesis efficiency as measured by light-induce chlorophyll fluorescence (F_v/F_m) and ion leakage reflected the level of cellular damage after chilling treatment at various temperature (0°C-6°C) for 7 days, F_v/F_m ratio decreased in the wild-type plants after 3 days, whereas, transgenic plants less affected. In order to dissect the hidden mystery, the *Arabidopsis COR* genes activated by *CBF1/DREB1B*, such as *KIN1*, *COR15a*, *COR47* and *RD29A*, that we used as probes did not cross-hybridize to transgenic tomato RNA even under low stringency conditions (unpublished data). It is possible that there are several *COR* homologs may present in the tomato genome, however, they are not very similar to *Arabidopsis COR* genes. Studies to elucidate the underlying molecular mechanism of abiotic stress tolerance in *Arabidopsis CBF1* overexpressing transgenic tomatoes and responsive genes are in their infancy. The question needs to be addressed is what are the other genes or enzymes involved in abiotic stress tolerance in transgenic tomato plants overexpressing *Arabidopsis CBF1* gene. In addition, we cannot rule out the possibility of existence of other homologous *COR* genes in the tomato genome and presumed to be function as a molecular shield for plant cells against freezing and drought stresses.

Oxidative Stress

As a consequence to environmental stresses increased generation of reactive oxygen species (ROS) occurs in plant cells [44,45]. This leads to the induction of protective mechanisms, including changes in gene expression, which lead to antioxidant activity, the recovery of redox

Table 1. *Agrobacterium*-mediated genetic transformation in tomato (*Lycopersicom esculentum*)

Explant type	*Preculture medium and duration*	*Duration of agro infection and co- cultivation*	*Selection medium*	*Transformation efficiency (TE) and remarks*	*Ref*
1	*2*	*3*	*4*	*5*	*6*
Leaf discs. cotyledons and hypocotyl segments	MS salts+B5 vitamins+3% sucrose -2 days	Gentle mix until leaf edges looks wet. -2 days	MS salts+BA 2.5 mg/l+IAA 1.0 mg/l+B5 vitamins	70% of plants are resistant to both Kanamycin and Nopaline. Inheritance of T-DNA was observed in T1 progeny by southern blot	[34]
Unpuntured and punctured cotyledons	TM-4G medium +Glucose 20 mg/l -2 days	Immersed in bacterial suspension -2 days	Hairy roots- hormone-free TM-4 and TM-5 medium. punctured cotyledons-TM-4G 2.4-D 0.5 μM	40-50% plants are resistant to Kanamycin	[58]
Cotyledons	Feeder layer plates prepared by tobacco cells on KCMS medium -One day	Immersed in bacterial suspension for 30 min. -2 days on MSO medium	MS salts+Myo-inositol 100 mg/l, Nitsch vitamin, Zeatin 2 mg/l+sucrose 3%	52% when cotyledons were incubated on tobacco feeder plates prior to co-cultivation. Acetosyringone did not significantly increased the transformation efficiency	[59]
Stem internodal segments	No preconditioning of explants	Upper cut surface of internode end pasted with agroculture -5 days	MSs1+Kn+claforan MSs2+Kn+claforan	30% of recovered plants are resistant to Kanamycin but sensitive to nopaline	[60]
Cotyledons	MSO medium +acetosyringone/ tomato wound factor	Exposed to diluted agroculture in MSO medium (1/10 MS salts) -one day	MS+ 3% sucrose	Highest TE (total % tumours per explant) was observed during first 24 hrs in the presence of acetosyringone/tomato wound factor	[61]
Cotyledons	Feeder layer plates prepared by tobacco cells on AB medium -One day	Immersed in bacterial suspension for 30 min. -2 days in dark	AZ medium (MS salts+ Zeatin 0.5 mg/l +IAA 0.5 mg/l+PVP 0.5 mg/l+3% sucrose	Use of chimeric GUS-intron gene to analyze early events of transformation. 15% of	[62]

Contd.

1	2	3	4	5	6
			First 72 hrs on medium C (MS salts+Zeatin 2 mg/l+IAA 0.1 mg/l (No kanamycin).	kanamycin resistant plants are recovered and confirmed by southern blot	
Cotyledons	Feeder layer plates prepared by Petunia cells on B medium -One day	Immersed in bacterial suspension for 20 min -2 days	After 72 hrs medium D (MS salts+Zeatin 2 mg/l+IAA 0.1 mg/l + kanamycin).	EHA 105 yielded 36% transformation frequencies and 80% of transgenic plants are diploid (=2n)	[63]
Cotyledon/ hypocotyl	Feeder layer plates prepared by tobacco cells on KCMS medium -One day	Explants were submerged in agro solution for 5 min. -2 days	Abaxial side of cotyledon in contact with 2Z medium. Rooting (modified MS with IAA 0.18 mg/l)	Factors affecting TE was studied and obtained 10.6% TE for moneymaker	[64]
Cotyledons	MS salts+B5 vitamins+3% sucrose -2 days	Gentle mix until explants wet. -2 days	MS salts+BA 2.5 mg/l+IAA 1.0 mg/l+B5 vitamins, Rooting medium (MSO+ IBA 2 mg/l+ kanamycin 100mg/l)	MP-1 line yielded 24 % TE than other lines	[65]
Cotyledons	Feeder layer plates prepared by tobacco cells on MSrk medium -2 days	Precultured explants were incubated with agrosuspension on a rotary shaker at 50 rpm for 30 min -2 days	MSc (MS salts+Zeatin 2.0 mg/l) MSt (MS salts+Zeatin 2.0 mg/l), Rooting MSr (MS salts+IAA 0.5 mg/l)	40% TE was obtained by using ticarcillin/potassium clavulanate antibiotic to eliminate *Agrobacterium*	[66]
Flamingo-bill explants		Explants were gently shaken in agro solution for 10 min. -2 days	MS basal salts+0.1 mg/l Zeatin (MSKC)	71% of regenerated shoots were positive for GUS and rooted normally	[67]

Contd.

1	2	3	4	5	6
Cotyledons	Feeder layer plates prepared by tobacco cells on KCMS medium -One day	Explants were sub-merged in agro solution for 5 min. -2 days	2Z medium Rooting (modified MS with IAA 0.18 mg/l)	Agrostrains MOG101 and UIA143 with additional *vir*G and *vir*G+*vir*E increased TE. BIBAC vector can be used to introduce high molecular weight DNA (150kb)	[68]
Cotyledons	Feeder layer plates prepared by tobacco cells on AB medium -One day	Explants were immersed in agro solution for 10 min. -2 days in dark at 28°C	AZ medium for bud initiation. MS119 medium for shoot elongation	TE was improved from 16-25% by using combinations of antibiotics.	[69]
Leaf. cotyledon and hypocotyl	MS salts+BA 1 mg/ l+NAA 0.1 mg/l. -One day	Explants were dipped in agro culture. -3 days	MS salts with different concentrations of IAA0.1 mg/l and Zeatin 2mg/l	20% of TE was achieved on MS salts+Zeatin 2 mg/l+IAA 0.1 mg/ l	[70]
Cotyledons	Adaxial parts of explants were placed on PCM medium. -2days in dark	Explants were sub-merged in agroculture consisting acetosyringone 200 iM for 8-10 min. 2-days in dark	SIM (MS salts+IAA 4mg/ l+Kinetin 4 mg/l+Zeatin 1mg/l)	1.8-11.3% TE, depending on genotype and procedure, 24.5-80% of transgenic plants are tetraploids	[71]
Cotyledon petiole, hypocotyl and leaf explants	No preculture	Excised explants were incubated in agro suspension for 7 min. -3 days in dark at 24°C	S/R1 medium (MS salts+B5 vitamins+Zeatin 1 mg/ l+IAA 0.01 mg/l+glucose 0.5%)	400 transgenic plants selected with 1% mannose in combination with 0.5% glucose and obtained 2-15.5% TE.	[72]
Hypocotyl	No preculture	Explants were immersed in Agroculture for 5 min. -3 days in dark	MS salts+2 mg/l 2.4-D+kinetin	Transgenic plants were established from hairy roots from 3 tomato cultivars with 25-65% regeneration frequency.	[73]

Contd.

1	2	3	4	5	6
Cotyledon	Explants incubated upside down in PM medium. -2 days in dark at 25°C	Incubated in bacterial suspension for 30 m; -2 days in dark at 25°C on tobacco feeder cell +CM medium with or without acetisyringone.	MS salts+Zeatin0.5 mg/l+IAA0.5 mg/l+Thiamine 0.4 mg/l	12.5% of TE was obtained on optimized conditions such as growth regulators. phenolic compounds and vitamins.	[74]
Cotyledons and hypocotyl segments	MS salts+B5 vitamins+3% sucrose -2 days	Explants were immersed in CT media. -2 days	MS salts+Zeatin 1 mg/l+IAA 0.1 mg/l+B5 vitamins	TE and regeneration was compared between solid and liquid medium. Liquid medium proved best for effective selection and positive regeneration	[75]
Cotyledons (MicroTom)	No preculture	The cotyledons were first placed adaxial side down on feeder plates and then poked gently with sharp forceps and incubated in agroculture for 10 min.	MS+Zeatin riboside 2mg/l+IAA 0.1 mg/l+B5 vitamins	57% average transformation frequency.	[1]
Cotyledons (MicroTom)	No preculture	Agro infection for 10 min.	MS+Zeatin 1.5 mg/l	TE exceeded 40% of the explants	[76]

BA, 6-benzylaminopurine; IAA, indol-3-acetic acid; NAA, α-naphthalene acetic acid; MS, Murashige and Skoog (1962) medium; Kn, kinetin.

balance, and recovery from damage or toxicity [44,45]. The ROS detoxification is relaying on scavenging antioxidants include ascorbate, glutathione, μ-tocopherol and carotenoids, as well as detoxifying enzymes namely superoxide dismutase (SOS), catalase (CAT) and enzymes of ascorbate-glutathione cycle prevent cellular structure damage [44,46]. Research carried out in the past few years has been generative in identifying transcription factors that are important for regulating plant responses to environmental stresses [44,47,48]. The heterologous transcriptional activator *CBF1* in tomato can regulate the expression of certain genes, which are involved in oxidative stress. To evaluate this hypothesis, subtractive hybridization has been used to isolate heterologous *DREB1B* responsive genes that may confer environmental stress tolerance in tomato plants [27]. The up-regulation of transcripts of *CAT1* (*CATALASE1*) gene and catalase activity in heterologous *DREB1B* transgenic plants in response to different stresses lead us to speculate cross talk between *CBF1* and several genes that function as stress protectants in tomato. In addition, transgenic plants were also exhibited, enhanced tolerance towards oxidative damage induced by the methyl viologen [27]. Evidently lower H_2O_2 content observed in transgenic tomato plants than controls, the catalase is considered to scavenge the H_2O_2 that is produced when plants respond to environmental stresses [49]. In maize, *CAT1* expression is induced by osmotic stress [50] and reported that *CAT*3 gene expression and its enzymatic activities are increased during acclimation in chilling-sensitive maize. In contrast, tomato plants overexpressing antisense *CAT1* gene are sensitive to chilling and oxidative stresses and provide direct evidence that *CAT1* plays an important role in protecting plants from these stresses [51].

Drought Stress

The DREBs/CBFs are encoded by multigene families, among them the *DREB1A* and *DREB2A* respond to low temperature and water deficit stresses, correspondingly [9]. Overexpression of *DREB1A* (*CBF*3) in transgenic *Arabidopsis* not only increases freezing tolerance but also drought and salt stresses [14]. The transgenic tomato plants overexpressing *CBF1* exhibited enhanced tolerance to drought in comparison to untransformed plants and is presumed to be the result of the responsive gene and its product and thought to function in protecting cells from water stress (Plate 1). Furthermore, higher survival rate, F_v/F_m, stomatal movement and proline content displayed that transgenic plants have the ability to cope with water deficit conditions better than control plants [28]. The mRNA level of *CAT1* gene and catalase activity increased in transgenic plants than controls, and implicate that upregulation of *CAT1* might be a consequence of the overexpression of *CBF1*. In addition, H_2O_2 concentration was also reduced in transgenic plants. The antioxidant enzymes such as glutathione reductase and superoxide dismutase activity increased in response to water deficit stress and overexpression of antioxidant genes improves tolerance to different biotic and abiotic stresses [52]. We speculate that there may be cross talk between *CBF1* and antioxidant enzyme gene(s), however, exact mechanism and novel antioxidant genes in tomato is needed to be investigated in detail.

Transgenic Tomato Plants Overexpressing *Arabidopsis CBF1* Exhibited Dwarf Phenotype

The tomato plants overexpressing *CBF1* driven by 35S promoter exhibited enhanced tolerance to chilling, oxidative and drought stresses, however, severe growth retardation of transgenic

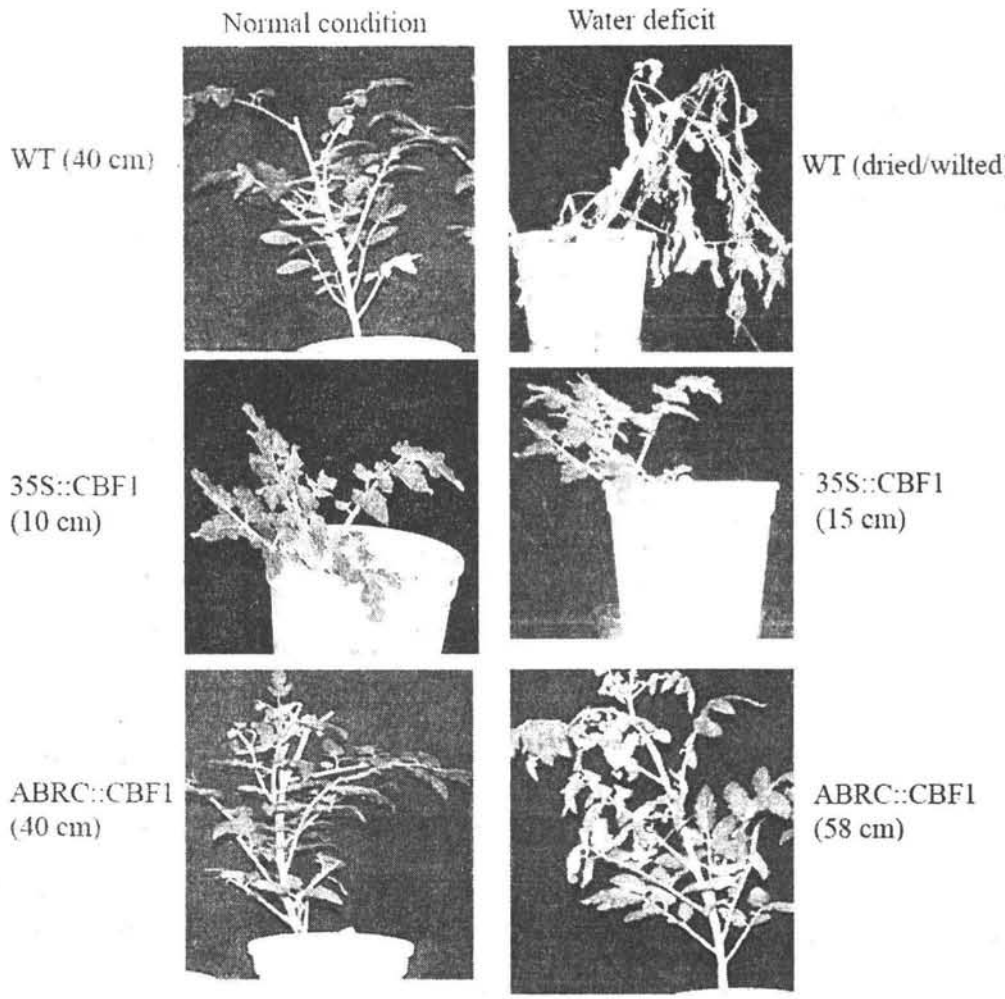

Plate I. Transgenic tomato plants expressing master switch Arabidopsis CBF1 gene driven by CaMV35S and ABRC promoter exhibited more resistance to water deficit than wild type (WT) (water deficit treatment = 28 days).

tomato plants observed and the average height of transgenic plants was less than 50% of that wild-type plant when comparing the phenotypes [27,28]. Similar effects were also observed in *Arabidopsis* plants overexpressing *DREB1A* [14], suggesting heterologous *CBF1* also affects developmental processes in transgenic tomato. In addition, transgenic tomato plants displayed decrease in fruit, seed number and fresh weight than wild-types under normal conditions. What might account for the apparent difference of the transgenic and wild-type tomato plants? However, this phenomenon reversed by GA_3 treatment, the seed number of transgenic plants treated with GA_3 did not be improved as compared to wild-type plants, but no obvious difference of fruit, seed production and fresh weights of transgenic tomato plants between normal or

water deficit conditions regardless of GA_3 treatment [27]. The possible reason may be *CBF1* protein interfering with GA_3 biosynthesis in 35S-*CBF1* transgenic tomato plants, however, needs to be investigated in detail.

Expression of *Arabidopsis CBF*1 Under the Control of Stress Inducible Promoter in Tomato

The transgene expression and patterns can be beneficially adjusted by using a promoter suitable for the plants background and for the type of transgene. For instance, the constitutive expression of *Arabidopsis CBF1* in tomato significantly improved tolerance to cold, oxidative and drought stresses at the expense of growth and yield under the normal conditions. Since, in this experiment tolerance was achieved at the expense of growth and yield, it was logical to assume to drive the expression of *Arabidopsis CBF1* with a stress inducible promoter to negate the side effects on the growth parameters of the transgenic tomato plants [14]. Henceforth, in order to improve agronomic performance, the stress inducible promoter, three copies of an ABA-responsive complex (ABRC1) from the barley *HAV22* gene, utilized to drive *Arabidopsis CBF1* [29]. The tomato plants overexpressing *CBF*1 under the control of ABRC1 stress inducible promoter exhibited enhanced tolerance to chilling, drought, and salt stress in comparison with untransformed plants and CaMV35S-*CBF1* tomato plants (Plate 1). The performance of the transgenic and untransformed tomato plants subjected to normal growth conditions after various stress treatments and non-stressed plants evaluated with respect to fruit number per plant, seed per fruit and fresh weight. As anticipated the transgenic tomato plants performed better than the untransformed plants under the tested stress conditions and the yield was better than the untransformed plants growing in similar conditions. Similarly, the transgenic *Arabidopsis* plants expressing the *DREB1A* under the control of stress inducible promoter RD29A not only exhibited enhanced tolerance to chilling, drought and salt stress but also solved the problem of growth retardation encountered in 35S-*DREB1A Arabidopsis* plants [14]. Further we propose that the *HVA22* gene may be one of the target genes of *CBF*1 protein, however, this hypothesis needs to be confirmed.

Conclusions and Future Prospects

Tomato is one of the important vegetable crops and has been the efforts of numerous biotechnologies during the past few decades. The integrated approaches of genetic engineering of tomato have been of great value because of its commercial values. Indeed, more recently tomato is the subject of new areas of research including functional genomics, proteomics and metabolomics. For the successful application of these new approaches to improve agronomic traits in tomato a reliable and reproducible transformation technique is essential. A new paradigm in the improvement of tomato and the demand created by the world's population is required to necessitating better management of natural resources by knowledge-based innovations in new technologies. Conventional breeding in tomato has made great progress in incorporating natural biotic and abiotic resistant genes, but the limitations of this method are also obvious. The progress in plant molecular biology now allows the generation of transgenic plants, thereby exploiting the mechanisms nature has developed to control and to limit the environmental effect on tomato crop. Changes in the expression or composition of

a desired gene may still be achieved by conventional breeding assisted by the results from transgenic research.

Although increased transformation efficiency would be desirable, several laboratories throughout the world have successfully produced transgenic tomato with a variety of introduced genes of novel agronomic traits [27-29,53-57]. Indeed, the initial focus has been primarily in the area of biotic and abiotic resistance, but as more potentially useful genes become available, we are likely to see the range of traits increase over the next several years. By considering advantages of *Arabidopsis CBF1* gene in tomato, similar strategies can be extended to other crops. However studies focusing on exact mechanism and regulation of *CBF1* and responsive gene(s) in tomato or in other crops are potential areas of research. Further research efforts to discover new 'master switch gene' like *CBF1* and engineering into economically important crops including tomato is beneficial, which can confer multiple-environmental stress resistance and are desirable for wide array of agroclimatic conditions.

Acknowledgements

Updates are not intended to be comprehensive reviews, and the authors apologize to the colleagues whose work could not be cited because of the restricted number of references. The authors thank Lee, J.T., Yu, S.J. and Hsieh, T.H., for photos. This work was supported by grants from Academia Sinica as well as National Science and Technology Program for Agricultural Biotechnology of the Republic of China.

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15 Late Embryogenesis Abundant (LEA) Protein Gene Expression and Regulation in Plant Stress Tolerance

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Introduction

Transgenic plants with enhanced stress tolerance play an important role in improving agricultural production and environmental industry. Different strategies can be used to enhance stress tolerance, such as the overproduction of compatible osmolytes, proline, glycine betaine, and mannitol, increases drought or high-salt tolerance [1-5]. Among these strategies, overexpression of genes encoding LEA protein, radical scavenging enzyme, and acyltransferase significantly improves drought or chilling tolerance [6,7]. Thus, it may be possible to develop tree and crop plants with enhanced resistance to various abiotic stresses through genetic engineering of *LEA* genes. Late embryogenesis abundant (LEA) proteins are hydrophilic proteins that accumulate to a high level in desiccation-tolerant tissues [8]. They are expected to play a protective role during dehydration; however, functional evidence is scarce.

It has been reported that LEA proteins have been localized in the cytosol and nucleoplasm [9,10], in chloroplast [11,12], in protein bodies and amyloplasts [13], and in vacuoles [14]. Heat-soluble dehydrin proteins have been detected in mitochondrial fractions from cereals [15]. In pea (*Pisum sativum*) mitochondria, a putative seed mitochondrial LEA-like protein (PsLEAm) exhibited sequence similarities with a soybean LEA protein has been identified [8]. The molecular cloning, subcellular localization, and expression profiling of the corresponding polypeptide are reported [8]. This protein could not be detected in vegetative tissues; however, its expression could be reinduced in leaves by severe water stress [8]. The recombinant PsLEAm was shown to protect two mitochondrial matrix enzymes, fumarase and rhodanese, during drying in an *in vitro* assay and is expected to participate in the protection of seed mitochondria against desiccation, allowing the rapid resumption of mitochondrial respiration during seed imbibition [8].

LEA protein expression often appears abscisic acid (ABA) dependent and can also occur

in vegetative tissues subjected to water deficit associated with drought, salt or cold stress [16-18]. Several mechanisms have been proposed to relate their structural features to the protection of cellular structures required by a dehydrated state: water replacement, ion sequestering, macromolecules, and membrane stabilization [18,19]. Recently, a LEA protein in pea seed mitochondria was identified through data mining [20]. The primary sequence of the protein that was called PsLEAm clearly revealed its LEA protein features and the presence of an N-terminal presequence that markedly diverged from the overwhelming hydrophilic profile of the mature protein [8]. To confirm the subcellular targeting of PsLEAm, a translational fusion of the transit peptide of PsLEAm with S65T-sGFP was transiently expressed in pea leaf protoplasts [8]. The reporter protein appeared clearly localized within mitochondria, indicating that the presequence of PsLEAm was a specific mitochondrial transit peptide [8]. Although some reports quoted the cold-increased accumulation of dehydrin-like protein in mitochondria from cereals, neither the nature nor the actual localization of the proteins was demonstrated [8,15]. In addition, Honjoh *et al.* [21] suggested that HIC6, a 14-kD Chlorella LEA protein, might be localized in mitochondria since the transgenic protein in tobacco (*Nicotiana tabacum*) seemed to cosediment with cytochrome oxidase in linear Suc gradient fractionation [8,21].

The most notable features of LEA are its enrichment in charged amino acids (40%) and its high-predicted content of α-helical structures [22,23]. Structure modeling of the tandem repeats of LEA revealed the remarkable conservation of the amphiphatic properties [8]. Although such a structure, which spans at least 120 residues, should be a key determinant for the function of the LEA proteins, LEA genes from different species may function physiologically and biochemical different. In this study, we overview the role of LEA including: (1) membrane stabilization, (2) improve salt and freezing tolerance, (3) desiccation tolerance, (4) bind calcium in a phosphorylation-regulated mode, and (5) iron transport.

LEA and Membrane Stabilization

Both the pattern of expression and the structural features of LEA proteins suggest a general protective role in desiccation tolerance [8,16,18]. In view of the apparent lack of well-ordered tertiary structure of LEA proteins preventing their use as catalysts, several mechanisms have been proposed to relate their structural features to the protection of cellular structures required by a dehydrated state: water replacement, ion sequestering, macromolecules, and membrane stabilization [8,18,19]. Dehydrins (DHNs; late embryogenesis abundant D-11) are a family of plant proteins induced in response to abiotic stresses such as drought, low temperature, and salinity or during the late stages of embryogenesis [24]. However, DHNs contain at least one copy of a consensus 15-amino acid sequence, which resembles a class A2 amphipathic α-helical, lipid-binding domain found in other proteins such as apolipoproteins and α-synuclein [24]. Koag *et al.* [24] showed that maize (*Zea mays*) DHNs can bind to lipid vesicles that contain acidic phospholipids and that DHN1 binds more favorably to vesicles of smaller diameter than to larger vesicles, and that the association of DHN1 with vesicles results in an apparent increase of α-helicity of the protein. Therefore, DHNs may undergo function-related conformational changes at the water/membrane interface, perhaps related to the stabilization of vesicles or other endomembrane structures under stress conditions [8,24].

Late-embryogenesis abundant (LEA) proteins accumulate to a high level in desiccation-tolerant tissues and are thus prominent in seeds [8,24]. They are expected to play a protective

role during dehydration; however, functional evidence is scarce [8,24]. Grelet *et al.* [8] reported that PsLEAm was localized within the matrix space of pea (*Pisum sativum*) seed mitochondria. PsLEAm revealed typical LEA features such as high hydrophilicity and repeated motifs, except for the N-terminal transit peptide [8]. Most of the highly charged protein was predicted to fold into amphiphilic α-helixes. PsLEAm was expressed during late seed development and remained in the dry seed and throughout germination [8,24]. The recombinant PsLEAm was shown to protect two mitochondrial matrix enzymes, fumarase and rhodanese, during drying in an *in vitro* assay [8]. LEA proteins in mitochondria have a beneficial role of protecting enzymes from drying during desiccation [8,24]. In addition, an iron-binding protein in the phloem of castor bean was identified as a LEA protein [25], and the ionic-binding properties of ERD14, an *Arabidopsis* dehydrin (group 2 LEA protein), were recently demonstrated and shown to depend upon phosphorylation, suggesting a role in calcium buffering [26].

LEA Improves Salt and Freezing Tolerance

Expression analysis of genes revealed that LEA has a typical seed profile with strong expression peaking during late maturation [8,27]. The expression of LEA was found to occur in response to severe dehydration in different plant species. Like many drought-induced genes, a number of LFA protein are known or expected to respond to the plant stress hormone ABA [8,18], and LEA protein mRNAs accordingly showed up in the transcriptome of ABA-treated plants [28]. However, it must be stressed that the ABA response involves complex and interacting signaling pathways [8,29]. LEA protein expression in response to ABA appeared not uniform and varied for individual members of the LEA family [8,30-32]. To investigate the role of the Vrn-1/Fr-1 intervals on the low-temperature inducibility of wheat Cor/Lea genes and its putative transcription factor gene Wcbf2, low-temperature response of these genes was monitored using near-isogenic lines (NILs) for the Vrn-1 loci [33]. The Wcbf2 transcript accumulated rapidly after low-temperature treatment and remained at a high level in lines without any dominant Vrn-1 alleles [33]. These results support the intimate relationship between the Cbf2/Cor/Lea expression and the level of freezing tolerance, and suggest that a functional Fr-A1 allele linked to the vrn-A1 allele, instead of the vernalization gene itself, plays a major role in regulating the CBF-mediated Cor/lea gene expression in wheat [33].

In *Arabidopsis*, gene expression during establishment, maintenance and release of dormancy is different in their degree of dormancy [34]. LEC1, FUS3 and ABI3, transcription factors are major regulators of embryo development and play some role in the control of dormancy [34]. Moreover, the late embryogenesis marker genes, AtEm1 and AtEm6, were reported in relation to the state of dormancy [34]. The expression of LEC1, FUS3 and ABI3 mRNA is only marginally different during seed development in various strong or moderate dormancy wild types, nsm mutants and abi3-1 [34]. ABI3 expression is influenced by cold and light, in a similar way in both dormant and nondormant wild-type seeds [34]. ABI3 transcript abundance in the nsm1 and nsm2 mutants is higher and in the nsm5-1 mutant is marginally lower than in wild-type seeds. The abundances of AtEm1 and AtEm6 mRNAs are equally affected by imbibition and cold temperature in mature and after-ripened seeds. The LEA transcript abundances for AtEm1 and AtEm6 are reduced in nsm mutants in a common, ABI3-independent pathway [34].

LEA Improves Desiccation Tolerance

Several reports demonstrated that the arbuscular mycorrhizal (AM) symbiosis is able to alter the pattern of dehydrin (LEA D-11 group) transcript accumulation under drought stress [8,35], and such a possible alteration functions in the protection of the host plants against drought [8,35]. Two dehydrin-encoding genes have been cloned from *Glycine max* (gmlea 8 and gmlea 10) and one from *Lactuca sativa* (lslea 1) and they have been analyzed for their contribution to the response against drought in mycorrhizal soybean and lettuce plants [35]. Results with soybean plants showed that most of the treatments did not show LEA gene expression under well-watered conditions. Only plants singly inoculated with *Bradyrhizobium japonicum* showed an important level of LEA gene expression under well watered conditions and a reduced level under drought stress conditions [8,35]. In lettuce, the lslea 1 gene was also induced by drought stress in all treatments. However, the level of induction was clearly higher in roots from non-inoculated plants than in roots from the two AM treatments assayed [8,35]. These results demonstrated that the levels of lea transcript accumulation in mycorrhizal treatments subjected to drought were considerably lower than in the corresponding nonmycorrhizal plants [8,35].

Late embryogenesis abundant (LEA) proteins, present in abundance in seeds during the late stages of development, are associated with desiccation tolerance [8,35]. Shih *et al.* [36] characterized a soybean LEA protein, GmPM16, with low molecular weight, high pI value, and an unusual amino acid residue distribution along the protein. The transcripts were detected in cotyledon mesophyll cells but not in the vascular system of mature or pod-dried soybean seeds [36]. Circular dichroism (CD) analysis and Fourier transfer infrared (FTIR) spectroscopy indicated that the GmPM16 protein in solution was highly unordered, possessing only partial α-helical structures [36]. However, the protein in sodium dodecyl sulfate (SDS) or trifluoroethanol (TFE) solution or in a dry state exhibited a conformation of abundant α-helical structures [36]. As well, the GmPM16 protein interacts with sugar and forms tightly glassy matrixes in the dry state [36]. The protein may play a role in reducing cellular damage in drying seeds by changing the protein conformation and forming tight cellular glasses [36].

LEA Binds Calcium in a Phosphorylation-regulated Mode

The ERD14 protein (early response to dehydration) is a member of the dehydrin family of proteins, which accumulate in response to dehydration-related environmental stresses [26]. Alsheikh *et al.* [26] show the *Arabidopsis* dehydrin, ERD14, possesses ion binding properties. ERD14 is an *in vitro* substrate of casein kinase II; the phosphorylation resulting both in a shift in apparent molecular mass on SDS-PAGE gels and increased calcium binding activity [26]. The phosphorylated protein bound significantly more calcium than the nonphosphorylated protein, with a dissociation constant of 120 μM and 2.86 mol of calcium bound per mol of protein [26]. ERD14 is phosphorylated by extracts of cold-treated tissues, suggesting that the phosphorylation status of this protein might be modulated by cold-regulated kinases or phosphatases [26]. Calcium binding properties of ERD14 purified from *Arabidopsis* extracts were comparable with phosphorylated *Escherichia coli*-expressed ERD14. Approximately 2 mol of phosphate were incorporated per mol of ERD14, indicating a minimum of two phosphorylation sites [26]. Western blot analyses confirmed that threonine and serine are possible phosphorylation sites on ERD14 [26]. Utilizing matrix assisted laser desorption

ionization-time of flight/mass spectrometry, we identified five phosphorylated peptides that were present in both *in vivo* and *in vitro* phosphorylated ERD14 [26]. These results suggest that the polyserine (S) domain is most likely the site of phosphorylation in ERD14 responsible for the activation of calcium binding [26].

LEA Increases Iron Transport

The transport of metal micronutrients to developing organs in a plant is mediated primarily by the sieve elements [8,25]. Ligands are thought to form complexes with the free ions in order to prevent cellular damage, but no binding partners have been unequivocally identified from plants so far [25]. Kruger *et al.* [25] used the phloem-mediated transport of micronutrients during the germination of the castor bean seedling to identify an iron transport protein (ITP). It is demonstrated that essentially all ^{55}Fe fed to seedlings is associated with the protein fraction of phloem exudate [25]. It is shown that ITP carries iron *in vivo* and binds additional iron *in vitro*. ITP was purified to homogeneity from minute amounts of phloem exudate using immobilized metal ion affinity chromatography [25]. It preferentially binds to Fe^{3+} but not to Fe^{2+} and also complexes Cu^{2+}, Zn^{2+}, and Mn^{2+} *in vitro*. The corresponding cDNA of ITP was cloned using internal peptide fragments. The deduced protein of 96 amino acids shows high similarity to the stress-related family of late embryogenesis abundant proteins [25]. Its predicted characteristics and RNA expression pattern are consistent with a function in metal ion binding. The ITP from *Ricinus* provides the first identified micronutrient-binding partner for phloem-mediated long distance transport in plants and is the first member of the late embryogenesis abundant protein family shown to have such a function [25].

Functional Genomics of LEA

Several LEA proteins have been shown to behave *in vitro* as cryoprotectants [10,37,38] and as stabilizers of glassy states [39]. Another LEA protein from maize, DHN1, was demonstrated to undergo conformational changes when binding specifically lipid vesicles, suggesting a role in membrane stabilization during stress [8,24]. By expressing the wheat LEA protein Em6 in yeast, Swire-Clark and Marcotte [39] brought to light the osmoprotective properties of the protein. However, although transgenic plants overexpressing LEA proteins exhibited improved stress tolerance [40,41], experimental evidence for the function of LEA protein remains faint [18]. More recently, a dehydrin was shown to bind calcium in a phosphorylation-regulated mode [26], and another LEA protein was assigned a role in iron transport in the phloem of *Ricinus communis* [8,25].

To understand the function of LEA genes in a genome scale, we conclude the sequences producing significant alignments after blasting of soybean (*Glycine max*) Late Embryogenesis Abundant (LEA) protein gene cDNA (U10111) through NCBI Blast (Table 1). Functions of several important LEA genes are included in Table 2. To understand the molecular mechanisms of cross talk between biotic and abiotic stress signaling pathways, we investigated the novel function of the *Tamarix androssowii* gene in drought stress tolerance transgenic pine (Figure 1). An overview of stress induced MnSOD gene expression and functions of overexpression of MnSOD in plant species, which are involved in membrane stabilization, improve salt and freezing tolerance, desiccation tolerance, bind calcium in a phosphorylation-regulated mode,

Table 1. Sequences producing significant alignments after blasting of soybean (*Glycine max*) Late Embryogenesis Abundant (LEA) protein gene cDNA (U10111) through NCBI Blast

Species and genotypes	*Locus of gene*	*Score (Bits)*	*E value*
Glycine max Essex dehydrin-like	U10111.1	1899	0.0
Vigna unguiculata dehydrin	AF159804.1	109	2e-20
Pisum sativum dehydrin 3 (Dhn3)	U91970.1	87.7	6e-14
Phaseolus vulgaris clone BAC-71F18	DQ323045.1	85.7	2e-13
Cicer pinnatifidum dehydrin 1 mRNA	AY170010.1	79.8	2e-11
Nicotiana tabacum NtERD10A mRNA	AB049335.1	71.9	4e-09
Lotus corniculatus var. *japonicus*	AP006422.1	63.9	9e-07
T. durum Desf. (Siliana) Dehydrin	X78431.1	61.9	4e-06
Maize RAB-17 gene	X15994.1	61.9	4e-06
Arabidopsis thaliana ERD10 AT1G2	NM180616.2	58.0	6e-05
Fagus sylvatica mRNA for dehydrin	AJ606474.1	58.0	6e-05
Medicago sativa dehydrin-like protein	AF411554.1	56.0	2e-04
Lavatera thuringiaca cold response	AF044584.1	56.0	2e-04
Hordeum vulgare subsp. *spontaneum*	AY349271.1	54.0	9e-04
Hordeum vulgare subsp. *spontaneum*	AY349248.1	54.0	9e-04
Arabidopsis thaliana glycine	L04173.1	54.0	9e-04
Arabidopsis thaliana AT5g66400/K1F13	AY093779.1	54.0	9e-04
Arabidopsis thaliana AT5g6640	AF428458.1	54.0	9e-04
Arabidopsis thaliana At1g11360/T23J18	AY050416.1	54.0	9e-04
Arabidopsis thaliana rab18 gene	X68042.1	54.0	9e-04
Zea mays mRNA for dehydrin	X15290.1	54.0	9e-04
T. aestivum L. mRNA for an ABA response	X59133.1	54.0	9e-04
Arabidopsis thaliana genomic DNA	AB013389.1	54.0	9e-04
Arabidopsis thaliana At5g66400/K1F13	BT002226.1	54.0	9e-04
Hordeum vulgare dehydrin	AF181459.1	54.0	9e-04
Zea mays PCO142314 mRNA	AY104757.1	54.0	9e-04
Hordeum vulgare dehydrin 9	AF043094.1	54.0	9e-04
Sorghum bicolor dehydrin	U63831.1	54.0	9e-04
Medicago truncatula clone mth2-32n7	AC170800.5	54.0	9e-04
Medicago truncatula clone mth2-22b18	AC144931.28	54.0	9e-04
Medicago truncatula clone mth2-9n11	AC141922.19	54.0	9e-04

and iron transport (Figure 2). In conclusion, LEA appears as the important protein identified from any organism. Its pattern of expression and its reported activity suggest that LEA could participate in stress tolerance and in the stabilization of mitochondrial matrix proteins in the dry state and hence contribute to desiccation tolerance of the seed [8]. Another interesting hypothesis based on computational analysis of LEA proteins suggested a structural role reminiscent of microfilaments for helical LEA proteins liable to form coiled-coils [27,42,43].

Table 2. Functional study of the Late Embryogenesis Abundant (LEA) protein gene in plant species

Species and genotypes	*Name of gene*	*Function*	*References*
Soybean [*Glycine max* (Gmlea 8 and Gmlea 10)]	Late Embryogenesis Abundant (LEA) protein gene	Results showed no LEA gene expression under well-watered conditions. The higher gene expression was found in non-inoculated plants subjected to drought. Only plants singly inoculated with *Bradyrhizobium japonicum* showed an important level of LEA gene expression under well-watered conditions and a reduced level under drought stress conditions.	[35]
Lettuce [*Lactuca sativa* (Lslea 1)]	Late Embryogenesis Abundant (LEA) protein gene	In lettuce, the *lslea 1* gene was also induced by drought stress and the level of induction was clearly higher in roots. The results demonstrated that the levels of LEA transcript accumulation in mycorrhizal treatments subjected to drought were considerably lower than in the corresponding nonmycorrhizal plants and the accumulation of LEA proteins is not a mechanism by which the symbiosis protects their host plant.	[35]
Soybean (*Glycine max* L.)	LEA protein, GmPM16	The GmPM16 protein interacts with sugar and forms tightly glassy matrixes in the dry state. The protein may play a role in reducing cellular damage in drying seeds by changing the protein conformation and forming tight cellular glasses.	[36]
Arabidopsis thaliana	LEC1, FUS3, ABI3 and Em	ABI3 transcript abundance in the *nsm1* and *nsm2* mutants is higher and in the *nsm5-1* mutant is marginally lower than in wild-type seeds, but changes due to temperature and light factors are very similar to those that occur in wild-type seeds. The abundances of *AtEm1* and *AtEm6* mRNAs are equally affected by imbibition and cold temperature in mature and after-ripened seeds. The LEA transcript abundances for *AtEm1* and *AtEm6* are reduced in *nsm* mutants in a common, ABI3-independent pathway.	[34]
Pea (*Pisum sativum*)	Late Embryogenesis Abundant (LEA) protein gene D	The recombinant *PsLEAm* was shown to protect two mitochondrial matrix enzymes, fumarase and rhodanese, during drying in an *in vitro* assay. The overall results constitute the first characterization of a LEA protein in mitochondria and experimental evidence for a beneficial role of a LEA protein with respect to proteins during desiccation.	[8]
Wheat (*Triticum aestivum*)	Late Embryogenesis Abundant (LEA) protein gene	These results support the intimate relationship between the *Cbf2/Cor/Lea* expression and the level of freezing tolerance and suggest that a functional Fr-A1 allele linked to the vrn-A1 allele, instead of the vernalization gene itself, plays a major role in regulating the CBF-mediated *Cor/lea* gene expression in wheat.	[33]

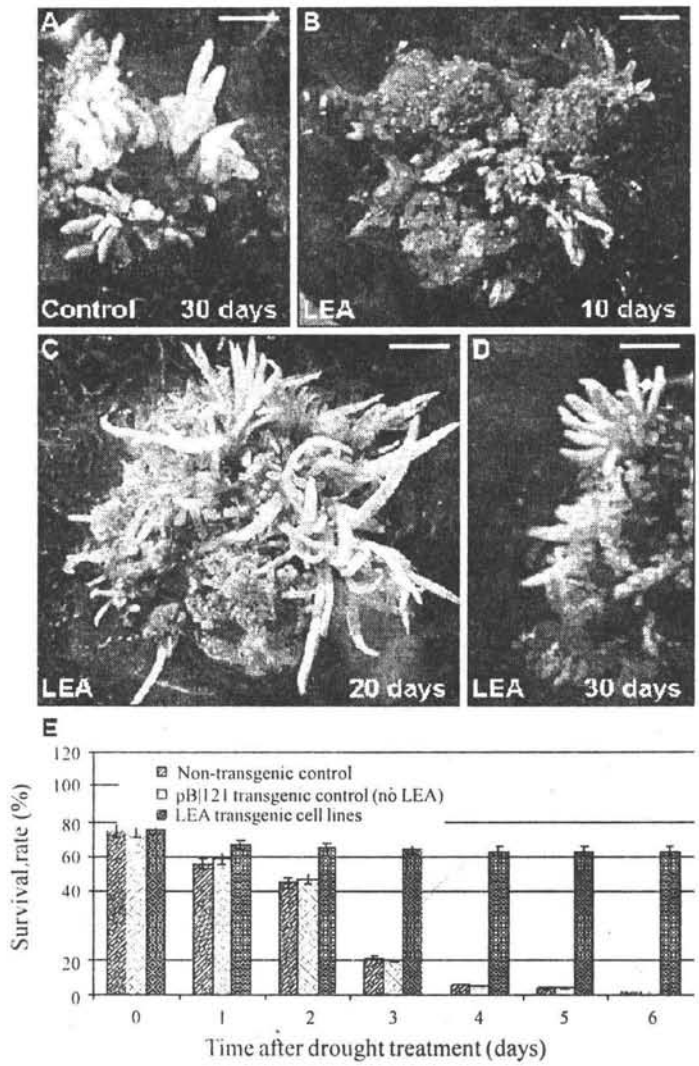

Figure 1. Drought tolerance of transgenic pine calli, shoots and plants overexpressing LEA. Callus cultures with shoots were dried for 10 days in Petridishes at room temperature and then returned to fresh medium for 30 days. (A) Non-transgenic control returned to fresh medium for 30 days after drought treatment for 10 days; (B) Transgenic shoots returned to fresh medium for 10 days after drought treatment for 10 days; (C) Transgenic shoots returned to fresh medium for 20 days after drought treatment for 10 days; (D) Transgenic shoots returned to fresh medium for 30 days after drought treatment for 10 days (bar = 0.5 cm); (E) Survival rate of plants derived from transgenic cell cultures. Survival rate was determined daily after drought treatments followed by returning to the normal growth environment. Photographs were taken at 10, 20 and 30 days after returning to the normal growth environment. The small bars indicate the standard errors.

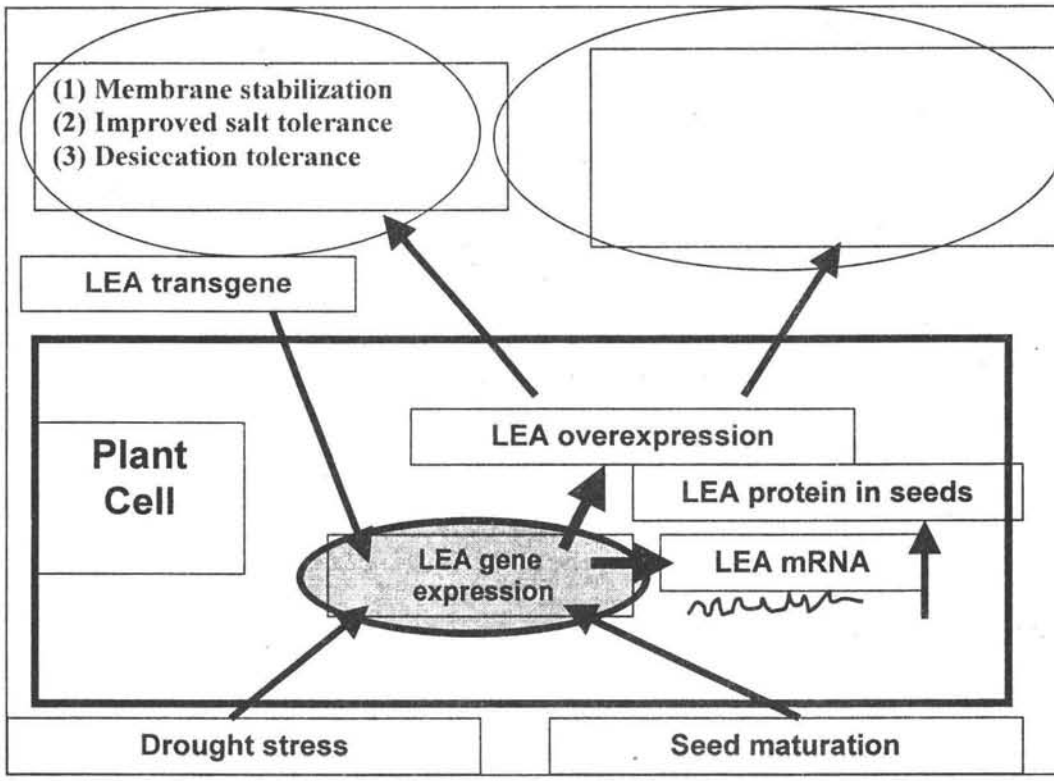

Figure 2. An overview of stress induced MnSOD gene expression and functions of overexpression of MnSOD in plant species, which are involved in membrane stabilization, improve salt and freezing tolerance, desiccation tolerance, bind calcium in a phosphorylation-regulated mode and iron transport. Biotic and abiotic stress such as drought, stress and freezing may induce expression of LEA in plant species through different pathways.

Owing to the early endosymbiotic origin of mitochondria [44] and to the probable coincidence of the evolution of desiccation tolerance with land colonization by primitive plants, it will be of interest to decipher the evolutionary history of LEA [8]. Seed desiccation and rehydration is accompanied by important changes in volume, also likely to involve mitochondria, and the reinforcement of the organelle structure by a filamentous network might be an asset. Progress has been made in stress tolerance biology [45-47] and genetic engineering of LEA genes will benefit to agricultural biotechnology worldwide.

Acknowledgements

The authors are grateful to Dr. Y.C. Wang for the LEA cDNA, and to undergraduate research assistants Karina Hesterberg, Ruba Sarsour, Adaeze Okoye, Hubert Chia and Zalak Daftary for their work in isolating mature embryos from seeds for callus induction. This work was supported by the East Carolina Christmas Tree Program.

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16 Triazole Induced Lipid Peroxidation and Antioxidant Defense Mechanisms in *Beta vulgaris* L.

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Introduction

Production of plants with tolerance to environmental stresses is one of the priorities in plant science research and certain chemicals have been demonstrated to regulate the expression of tolerance [1,2].

Tuber crops become the most important food crops after cereals and legumes. They form a rich source of energy for people living near sustenance level in Tropical East and West Africa, East and South Pacific Islands and part of South America. Beet root (*Beta vulgaris* L.) is one of the important vegetable crops available at low cost to the poor people in India. It belongs to the family of chenopodiaceae. It has been known for a long time in ancient Greece and Rome. During Napoleonic wars, France was cut off the supply of sugar and Napoleon (1811 A.D.) encouraged the cultivation of *Beta vulgaris* for sugar manufacturer in France. It has become an important crop in the 19th century. It was now extensively cultivated in Europe and to considerable extent in the USA and New Zealand [3]. It is a root crop suitable for growing both in cool and warm climate. The leaves and tubers are used as green salad, boiled vegetable and also for pickling. Roots are a good source of sugar and also can be used for canning. It also serves as a rich source of nutrition for human and animals, and as commercial feedstock for many industrial applications.

Over the past two decades, triazole groups of fungicides have become increasingly important due to their wide spectrum of action and their low application rate, mainly caused by high agropetal systemic properties and vapour phase activity [4]. Triazole compounds such as triadimefon, hexaconazole, uniconazole and paclobutarol etc. are widely used as fungicides and they also possess varying degree of plant growth regulating properties mediated by their interference with the isoprenoid pathway and subsequent shift in the balance of important plant hormones including GA, ABA and cytokinins [5].

Plant growth retarding activity of triazole compound was first described for triadimefon and triadimenol [6]. Later, other type of triazoles, which reduce growth in plants were described and various compounds such as paclobutrazol, triapenthenol, uniconazole etc. have been used in several studies [7-10]. Triazoles inhibit gibberellin and ergosterol biosynthesis both in plants and fungi respectively [11]. They also induce a variety of morphological and biochemical responses in plants for e.g. paclobutrazol induced tuber initiation and enlargement in potato [12-14]. The primary site of plant growth retardant activity is inhibition of the activity of ent-kaurene oxidase, the enzyme that catalyzes the sequential oxidation of ent-kaurene to ent-kaurenoic acid, the early steps in the biosynthetic pathway to GA_3 [15-17]. Triazole compounds like triadimefon, paclobutrazol, uniconazole, propiconazole and hexaconazole also have growth regulating properties and induced many morphological and metabolic changes like reduction in shoot elongation, stimulation of rooting, inhibition of gibberellin biosynthesis, increased chlorophyll content, altered carbohydrate status and increased cytokinin synthesis [18-20].

Regulation of the enzymes and products in the gibberellin biosynthetic pathway by triazole has been reviewed by Graebe [21] and Hedden and Kamiya [22]. Several triazole compounds protect plants from temperature, drought and other stresses [23,24]. An increase in cytokinin caused by triazole treatment could lead to enhanced chloroplast size and chlorophyll levels [1]. The level of *cis*-abscisic acid was found to be affected by triazole application to plants. One of the hypothesis put forth to explain the mechanism of triazole induced increase in plant stress resistance in that, these compounds are initiating the programmed hardening process involving the accumulation of ABA [19].

Triazoles affect the activities of several other enzymes by relating to detoxification of active oxygen species and antioxidant metabolism [25-28]. Fletcher *et al.* [1] reviewed the role of triazoles enhancing antioxidant activity and thus protecting the plant from various environmental stresses. The main objectives of this study are to assess the effect of triadimefon and hexaconazole on lipid peroxidation and antioxidant potential of *Beta vulgaris* L. cv. Mahycolal-II.

Materials and Methods

Seeds of *Beta vulagris* L. cv. Mahycolal-II obtained from Maharashtra Hybrid Seed Company Limited (MAHYCO), Maharashtra, India, were used and the experiments were conducted at the Botanical Garden of the Annamalai University, India.

The seeds were treated with 0.2% mercuric chloride solution for 5 min with frequent shaking and thoroughly washed with deionised water to remove the traces of mercuric chloride. The beet root seed balls were directly sown in the plots of 3X1 m, filled up to 50 cm depth with red soil, sand and farm yard manure (FYM) at the ratio of 1:1:1. Seed balls were sown at a spacing of 20 cm between rows and 10 cm between seeds, irrigated with tap water (pH 6.8) up to 60 DAS and care was taken to avoid excess irrigation. The field were irrigated with hexaconazole (5 mg L^{-1}), triadimefon (15 mg L^{-1}) as triazole compounds, and water as control, on 12, 24, 42 and 57th days after sowing (DAS).

In the preliminary experiments, 2, 5, 10, 15, 20 and 25 mg L^{-1} concentrations of hexaconazole [Imperial Chemical Industrial, England] and triadimefon [Bayer India Limited] from commercial preparations were used for treatment to determine the optimum concentration

of these compounds. Among these concentrations, 5 mg L^{-1} of hexaconazole and 15 mg L^{-1} of triadimefon were found to increase the dry weight significantly and higher the concentration, slightly decreased the growth and dry weight. Hence, the above concentrations were used to determine the effect of these triazole compounds on lipid peroxidation and antioxidant defense in *Beta vulgaris* L. cv. Mahycolal-II.

During the experimental studies, the temperature was 21.75-28.68°C. Average rainfall was 550 mm and the average relative humidity (RH) was 87 per cent, from November to February during 2001–2002 and 2002-2003. Plants were harvested randomly on 15,30,45 and 60th DAS and washed with tap water followed by treatment with deionised water. The plants were then separated into shoot (leaves) and tuber for further analysis.

Lipid peroxidation was assayed with the reaction mixture of thiobarbituric acid (TBA, 0.5%) and TCA (20%) as per the protocol of Heath and Packer [29]. Carotenoid contents were extracted from the third leaf discs (0.8 cm in diameter, except the midrib) and in the periphery of the tuber tissue (0.5 cm from the epidermis and 1 cm from the top of root tuber) [30]. The content of the same was determined spectrophotometrically according to the formula of Kirk and Allen. Ascorbic acid was extracted and estimated by the method of Omaye *et al.* [31]. Reduced glutathione (GSH) was assayed according to the procedure of Griffith [32]. The activities of superoxide dismutase (SOD) [33], peroxidase (POD) [34], ascorbate peroxidase [35] and catalase (CAT) by the method of Chandlee and Scandalios [36] with slight modifications, were determined according to standard procedures.

The experiments were laid out in a Completely Randomized Block Design (CRBD) with 21 replicates for each treatment. The analysis of variance (ANOVA) was conducted using the method of Ridgman [37]. Means were compared between treatments from the error mean square by LSD (Least Significant Difference) at the $P < 0.05$ and $P < 0.01$ confidence level using Tuckey's test [38].

Results and Discussion

An investigation was carried out with an objective of estimating the lipid peroxidation and antioxidant properties of the triazole compounds viz. triadimefon and hexaconazole on *Beta vulgaris* L.

Triazole treatments were significantly inhibited the lipid peroxidation of membrane of the leaf tissue. Among the triazole treatments, there was no significant difference in the lipid peroxidation between triadimefon and hexaconazole treated plants and it was 80.06 and 79.34 per cent over control respectively on 60 DAS (Figure 1A). In the case of tuber tissue, triadimefon and hexaconazole inhibited lipid peroxidation in a larger extent when compared to control on 60 DAS (85.02 and 81.25% respectively; Figure 1B). The triadimefon and hexaconazole treated plants showed a lower level of lipid peroxidation in *Beta vulgaris* when compared to control. Lipid peroxidation is measured by the malonoldialdehyde (MDA) released [39], which is a consequence of higher oxidative stress [40]. Similar results were observed in triazole treated *Egeria densa* leaves [41,42] and paclobutrazol treated wheat seedlings [43].

Triazole treatments significantly increased the carotenoid content to a larger extent. Among the triazoles, hexaconazole increased the carotenoid content to 159.349 per cent over control on 60 DAS, which was higher than that of triadimefon treated plants (123.632

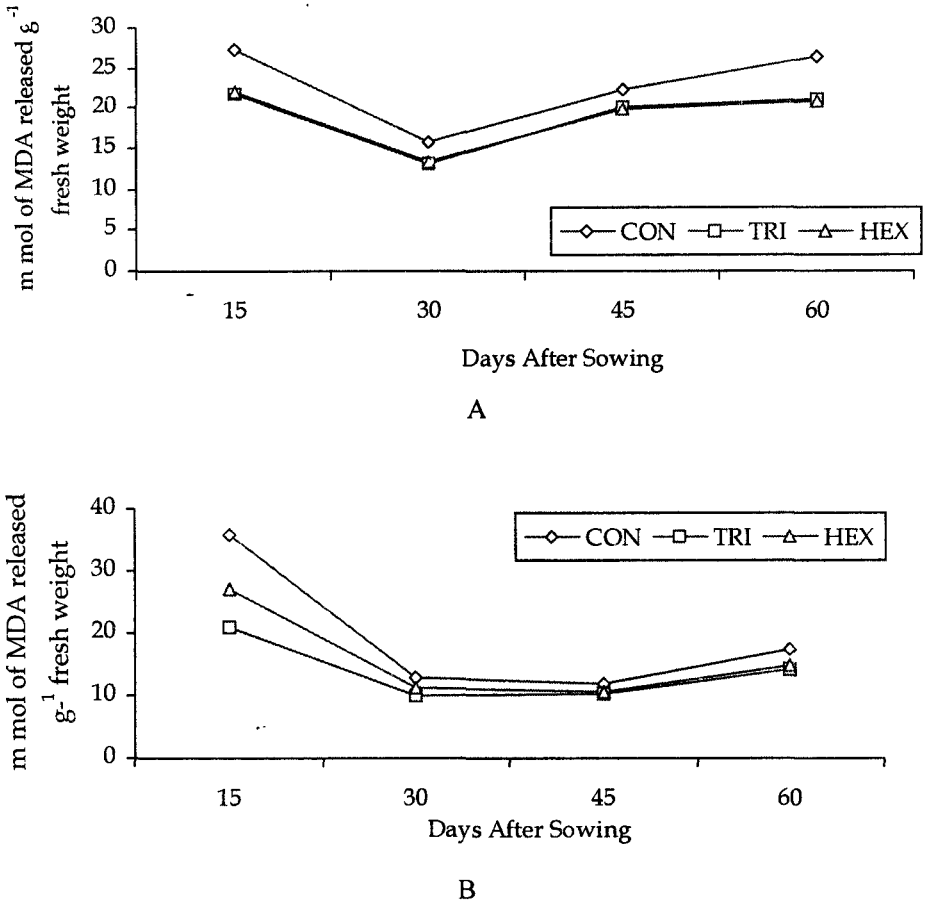

Figure 1. Triadimefon and hexaconazole induced changes in the lipid peroxidation of beet root leaf (A) and tuber (B) tissue.

per cent). Triazole treatment increased the carotenoid content to a larger extent in the tuber tissue when compared to untreated control. Among the triazoles, triadimefon induced a higher level of carotenoid content than hexaconazole in the tuber tissue (Figure 2B). However, hexaconazole increased it to a higher level in leaf tissue (Figure 2A). Among the organs tuber showed highest carotenoid content in the plants. Triadimefon treatment induced higher level of carotenoid content in barley [6], cowpea [44] and cucumber cotyledons [45]. Similar results were observed in uniconazole treated wheat seedlings [19], tomato [46], dichlorobutrazole treated *Zea mays* [47] and paclobutrazol treated wheat seedlings [43]. Carotenoids are involved in the protection of the photosynthetic apparatus against photoinhibitory damage by singlet oxygen ($^1O_2$) that is produced by excited triplet stage of chlorophyll thus indirectly reducing the formation of reactive oxygen species in wheat [48,49]. Increased level of cytokinin particularly transzeatin and its riboside has been reported in sunflower cell suspension, rice,

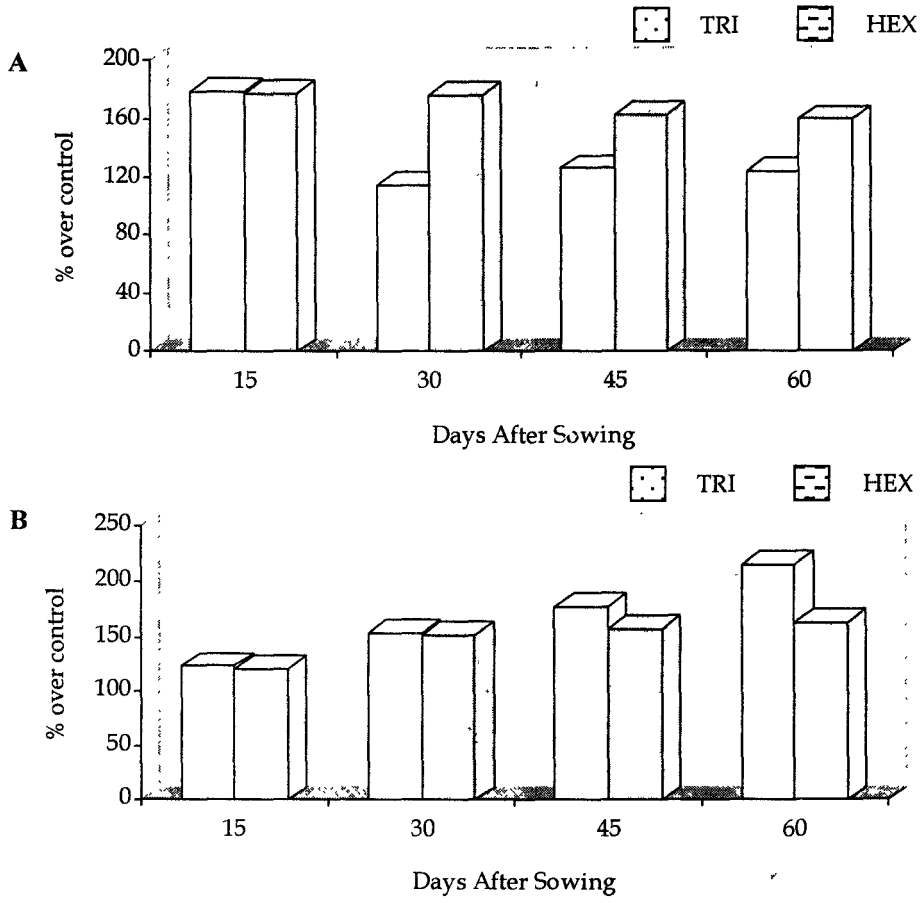

Figure 2. Triadimefon and hexaconazole induced changes in the carotenoid content of beet root leaf (A) and tuber (B) tissue.

soybean and rape seedlings after uniconazole treatment and thus increased zeatin might be responsible for the increased synthesis of carotenoid in these plants [50-53].

In the leaves, the ascorbic acid content was increased with age in the control and treated plants. Among the triazoles, hexaconazole treatment increased the ascorbic acid content in both leaves and tubers to a level higher than that of triadimefon (Figure 3A). Similar results were obtained in the tubers at all stages of growth. Triazole treatment significantly increased the ascorbic acid content in the tubers when compared to leaves (Figure 3B). Ascorbic acid is an important component of the plant antioxidant system [54-56] and it has been proposed to have roles in relation of photosynthesis [55], cell expansion [54] and *trans*-membrane electron transport [57]. Ascorbic acid also has another important photoprotective function because of its antioxidant capacity [58]. Uniconazole increased the level of the antioxidants like, α-tocopherol and ascorbic acid in tomato seedlings, and protect membrane by preventing or reducing oxidative damage [46]. Singh [59] reported an increase in ascorbic acid content in

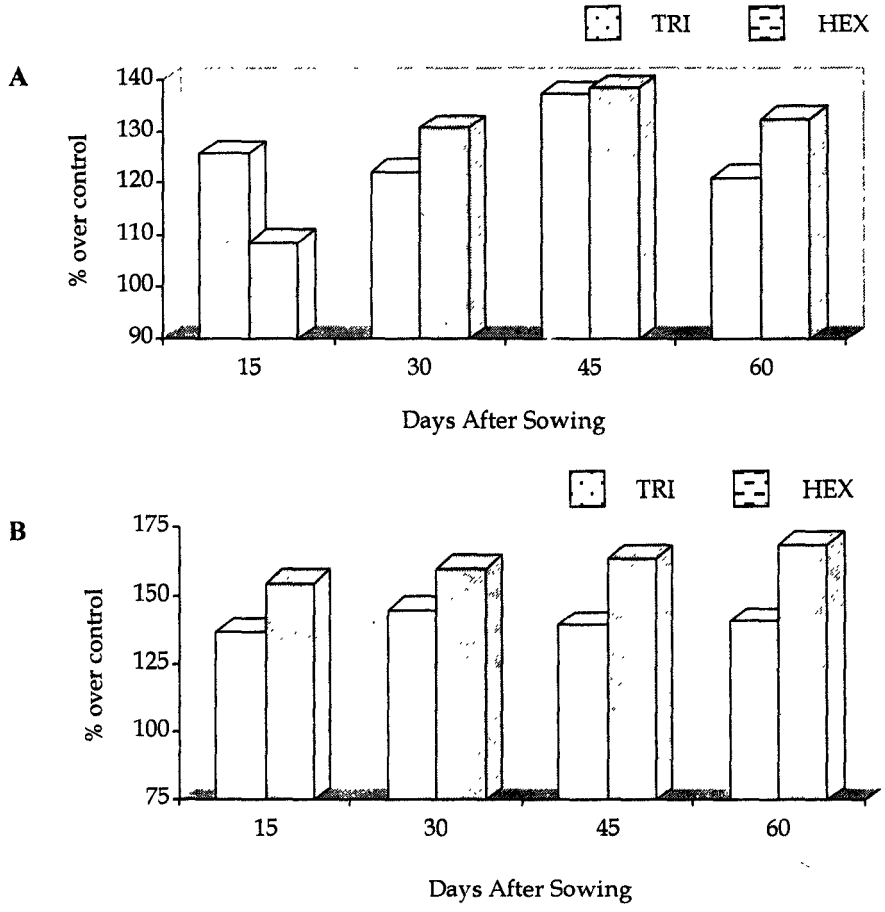

Figure 3. Triadimefon and hexaconazole induced changes in the ascorbic acid content of beet root leaf (A) and tuber (B) tissue.

the fruit juice of paclobutrazol treated mango (*Mangifera indica* L). Similar results were observed in grape fruits [60] and *Citrus limon* [61].

The reduced glutathione content increased with age in the leaves of treated and control plants. The reduced glutathione content of the leaves significantly increased to 116.719 and 140.132 per cent over control in the triadimefon and hexaconazole treatments respectively on 60 DAS (Figure 4A). Triazole treatment increased the reduced glutathione content of the tuber at all stages of growth and it was 122.428, 130.161, 131.394 and 132.265 per cent over control for triadimefon and 126.981, 133.236, 136.671 and 136.859 per cent over control for hexaconazole respectively on 15,30,45 and 60 DAS. Among the triazoles, hexaconazole treatment increased the reduced glutathione content in the tuber to a higher level than that of triadimefon (Figure 4B). Both triadimefon and hexaconazole treatment significantly increased the reduced glutathione (GSH) content to an appreciable level. Similar observations have been

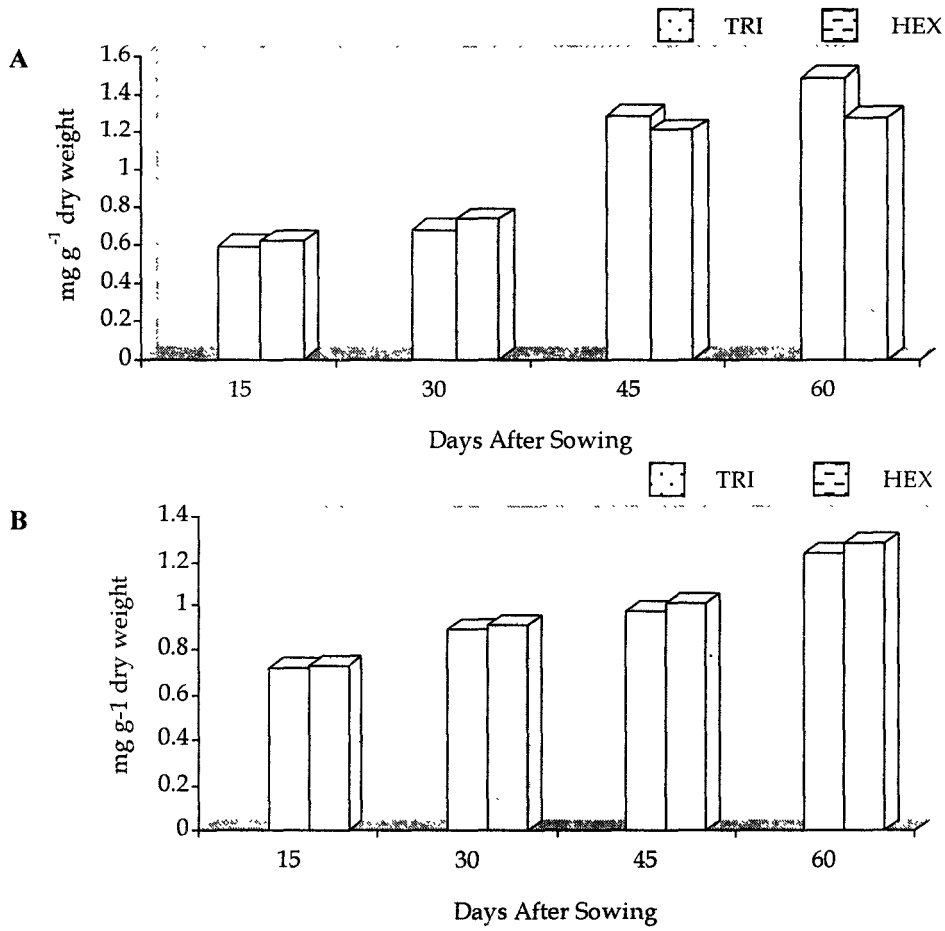

Figure 4. Triadimefon and hexaconazole induced changes in the reduced glutathione content of beet root leaf (A) and tuber (B) tissue.

made in wheat cultivars [62] and leaves and roots of bean [63,64]. Glutathione is regenerated by the action in GR (Glutathione reductase) in a NADPH-dependent reaction [65]. Ascorbate reductase catalyses the conversion of monodehydroascorbate or dehydroascorbate by oxidizing glutathione [65]. The glutathione pool is an important redox components in plant cells. Changes in intracellular glutathione status may therefore, be expected to have important consequence for the cell, through modification of the cell metabolic functions associated with glutathione regulated genes [66]. Antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase and non-enzymatic antioxidants such as glutathione play a major role in protection of cell against ionizing radiation-induced damage [67]. Reduced glutathione participates non-enzymatically in protection against toxic compounds [68,69].

The activity of superoxide dismutase (SOD) of the leaves increased with the age of the plant. Triazole treatment increased the level of superoxide dismutase activity significantly when compared to control. Among the triazoles, hexaconazole treated plants showed higher

level of superoxide dismutase activity than that of triadimefon and it was 126.754 and 105.132 per cent over control respectively on 60 DAS (Figure 5A). The superoxide dismutase activity in the tuber increased with the age of the plant (Figure 5B). Triazole treatment showed a higher level of superoxide dismutase activity in the tubers when compared with control and it was 126.001, 108.413, 116.764 and 123.435 per cent over control for hexaconazole and 104.001, 109.760, 112.634 and 120.634 per cent over control for triadimefon respectively on 15,30,45 and 60 DAS. Among the organs, tuber showed higher level of superoxide dismutase activity when compared to leaf. Superoxide dismutase activity increased with triazole treatments to a level higher than that of control plants. Superoxide dismutase play a major role in combating oxygen radical mediated toxicity [70]. High level of superoxide dismutase activity in the tissue of *Mangifera indica* L. (Mango fruit) retarded the membrane permeability [71,72]. Paclobutrazol treatment increased the activity of superoxide dismutase, glutathione reductase and ascorbate

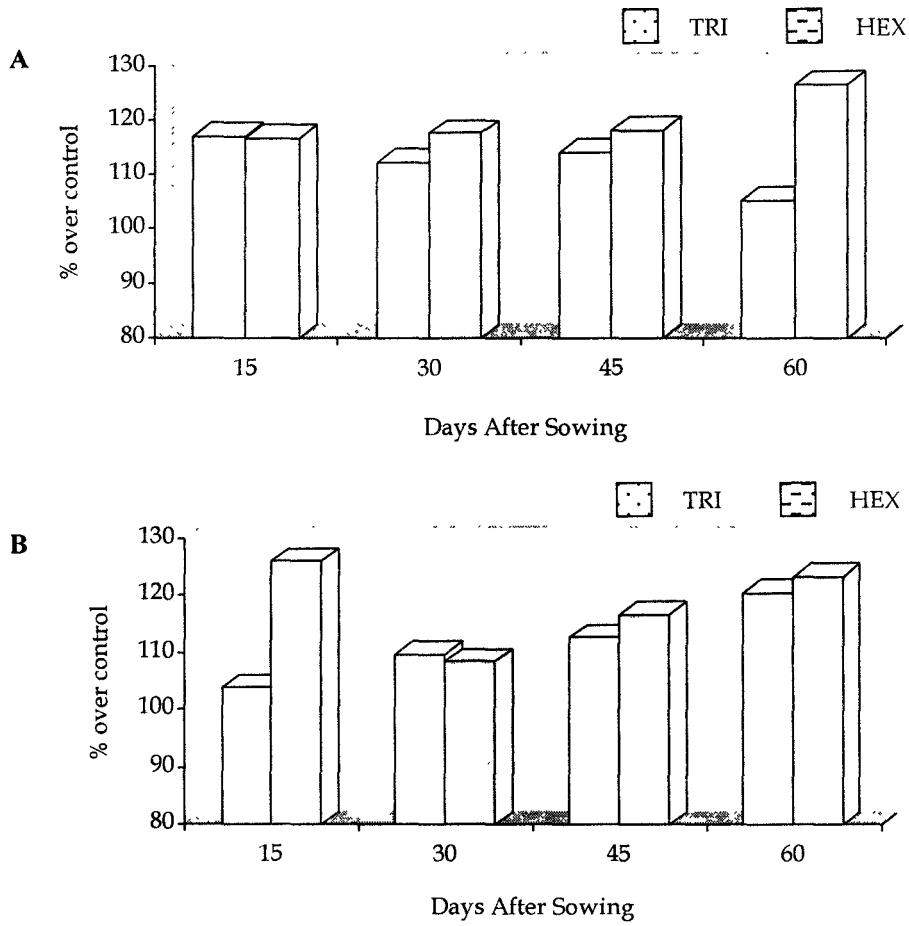

Figure 5. Triadimefon and hexaconazole induced changes in the superoxide dismutase activity of beet root leaf (A) and tuber (B) tissue.

peroxidase in the leaves and roots of bean [63,64], wheat [28,62], banana [73] and maize seedlings [74]. Uniconazole treatment protected corn seedlings from damage and the stress protection is mediated by an increased activity of antioxidant enzymes [75]. Similar observations were made in grape seedlings [76] and cucumber [77].

In the leaves of beet root, peroxidase (POD) activity increased with age of the plant. Triazole treatment increased the peroxidase activity to a larger extent when compared to control. Among the triazole treatments there is no significant variation in peroxidase activity in the leaves (Figure 6A). In the tubers, the peroxidase activity was increased with the age up to 45 DAS, later it declined (Figure 6B). Among the plant organs, leaf tissue showed slightly higher activity than tuber tissue. Treatment with triazole compounds increased the peroxidase activity in the leaves and tubers of beet root and it was very high in the leaves when compared to tuber tissues. An increase in peroxidase activity is a common response to oxidative and abiotic

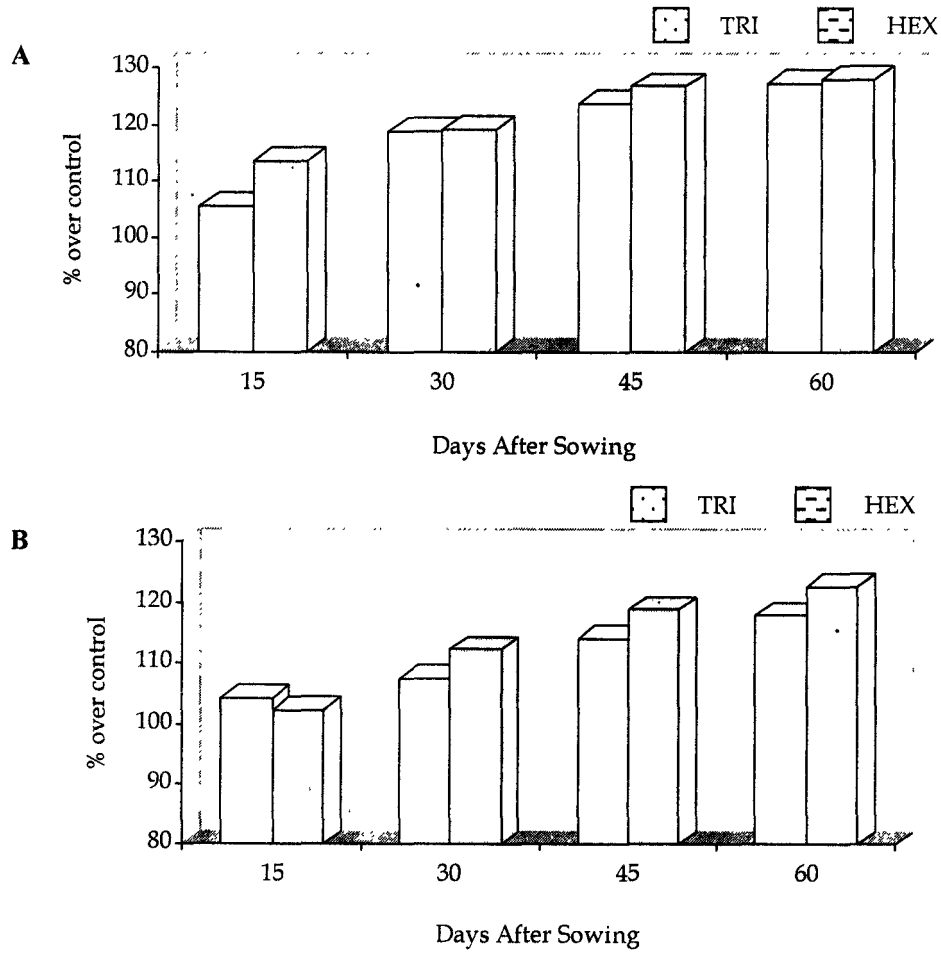

Figure 6. Triadimefon and hexaconazole induced changes in the peroxidase activity of beet root leaf (A) and tuber (B) tissue.

stresses. Therefore peroxidase could be part of the enzymatic system connected with the increase in ethylene formation in spinach plants [78]. Enhanced production of oxygen free radicals is responsible for peroxidation of membrane lipids and the degree of peroxidative damage of cells is controlled by the potency of antioxidative peroxidase enzyme system [78]. Triadimefon increased the level of peroxidase activity in black gram [79], bhendi [80] and *Raphanus sativus* [81]. Similar results were observed in paclobutrazol treated wheat cultivars [28], wheat seedlings and bean [28,43,62-64]. Hence, treatment with triazole compounds increased the superoxide dismutase and peroxidase activity in *Beta vulgaris* indicating the co-ordinated activity of these enzyme in scavenging the reactive oxygen species in beet root.

The ascorbate peroxidase (APX) activity increased with the age in the leaves and tubers of beet root (Figure 7A,B). Triazole treatment significantly increased the ascorbate peroxidase activity to an appreciable level. Among the triazole treatment, hexaconazole treatment increased it to a higher level than that of triadimefon. The ascorbate peroxidase activity was higher at the time of tuber maturation. The ascorbate peroxidase activity increased in the triazole treated *Beta vulgaris* plants when compared to the control. Similar observations were made in paclobutrazol treated *Echinochloa frumentacea* [25] and wheat seedlings [28,62], and triadimefon treated cucumber seedlings [45]. Ascorbate peroxidase utilizes ascorbic acid as an electron donor in the neutralization of H_2O_2 both in the cytosol and in molecular compartments [55]. Ascorbate also scavenges O^-_2 and $^1O_2$ reduces through the action of ascorbate

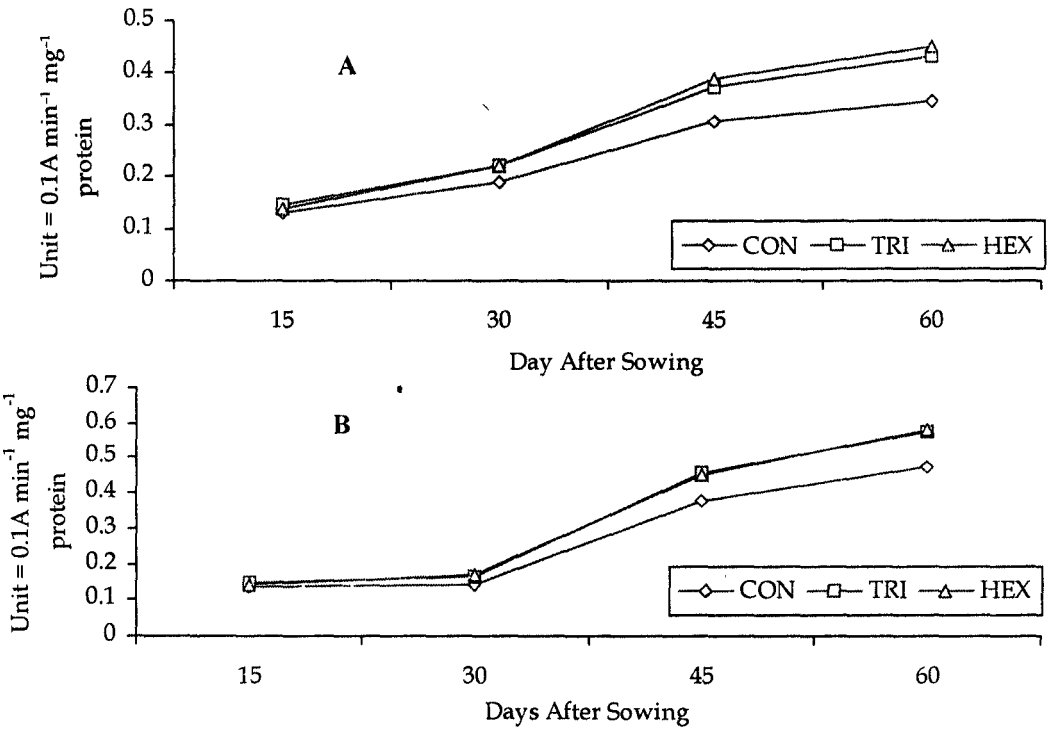

Figure 7. Triadimefon and hexaconazole induced changes in the ascorbate peroxidase activity of beet root leaf (A) and tuber (B) tissue.

peroxidase [65]. Ascorbate peroxidase represent the major enzymes of hydrogen peroxide (H_2O_2) degradation and it is interesting to note that the apoplast of barley leaves has been shown to contain substantial amounts of superoxide dismutase, ascorbate peroxidase required for destruction of superoxide and H_2O_2 [82]. Paclobutrazol stimulated increase in the activities of superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR) [28]. Some of the free radical scavenging enzymes has been reported to increase in wheat [28], corn [75] and cucumber [77] plants after triazole treatments. The triazole compounds enhance the free radical scavenging capacity in treated plants including the levels of carotenoids, ascorbate, superoxide dismutase and ascorbate peroxidase [46,62].

In the leaf tissue, the catalase (CAT) activity increased with the age in the control and treated plants. Triazole treatments significantly increased the activity of catalase to a higher extent when compared to control. Among the triazoles, hexaconazole treatment increased the catalase activity to a larger extent when compared to triadimefon and it was 132.75 and 129.66 per cent over control on 60 DAS respectively (Figure 8A). In the tuber tissue, catalase (CAT) activity increased with the age of the plant. Triazole treatments significantly increased the catalase activity to a level higher than that of control (Figure 8B). Among triazoles, triadimefon

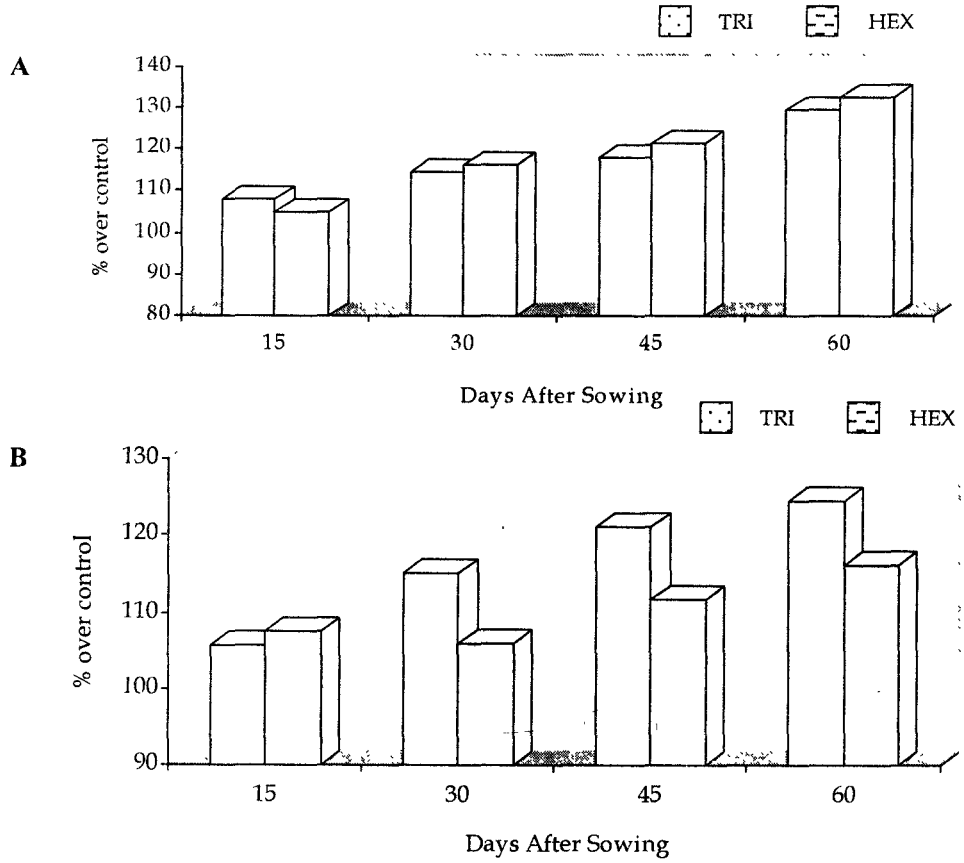

Figure 8. Triadimefon and hexaconazole induced changes in the catalase activity of beet root leaf (A) and tuber (B) tissue.

treated plants showed higher level of catalase activity when compared to hexaconazole in tuber tissue. Catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX) appear to play an essential role in the scavenging processes when co-ordinated with superoxide dismutase (SOD) activity [83]. Catalase represents the major enzyme of H_2O_2 degradation [82] as reported in wheat [84]. Increase of catalase activity was observed under drought and chilling in maize [85] and tomato seedlings [86]. An increase in the activity of catalase was also observed in all the mulberry genotypes on high temperature treatment [65]. Triadimefon increased the catalase activity significantly in cucumber seedlings [45]. ABA increases the activities of antioxidant enzymes such as superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase (GR) in plant tissues under water stress [87-91]. Triadimefon and hexaconazole treatments have resulted in the increase in activities of superoxide dismutase and catalase. Induced activity of two enzymes by these triazoles may confer a high antioxidant protection of *Beta vulgaris* plants.

Conclusion

Plant growth regulators play an important role in modifying plant structure and partitioning photosynthates to the economically useful organs of plant. Triazole compounds are widely used as systemic fungicides to control diseases in plants and they have both fungitoxic and plant growth regulating properties. These compounds also improved the partitioning of photosynthates more to the root system than to the shoot and this character can be exploited to improve the yield in the tuber crop. Triadimefon and hexaconazole are widely used triazole fungicides.

The lipid peroxidation was inhibited by the triadimefon and hexaconazole and the inhibition was high in the leaves than tuber tissues. Triazole compounds increased the carotenoid content to a larger extent. Among the organs, tuber showed higher carotenoid content than leaves. The major water-soluble antioxidants, ascorbic acid and reduced glutathione content significantly increased with triazole treatment when compared to control. The ascorbic acid content was higher in the tuber when compared to leaves of beet root.

The activities of enzymic antioxidants like superioxide dismutase, peroxidase, ascorbate peroxidase and catalase were increased to higher level by triazole treatments. These antioxidant enzymes showed an elevated level of activity in the tubers when compared to the leaves in *Beta vulgaris* L. Based on the results of the experiments conducted, triazole treatments lowered the membrane damage by inhibiting lipid peroxidation and caused increase in carotenoid contents. Antioxidant potential was also increased by triazole treatments as supported by the increase in non-enzymic and enzymic antioxidants.

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17 Genetic Diversity and Development of Aluminium Tolerance to Abiotic Stresses in Crop Plants of Portuguese Archipelago of Madeira

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Introduction

Global climatic and adverse environmental changes including drought, rising atmospheric CO_2 levels and acidification of soils have been accelerated dramatically in the past few decades. These changes have profound effects on the performance of modern agricultural crops that have potential to produce high yields but only under intensive management practices. At the same time, a growing interest in the diversification of food sources and increasing consumption of functional foods have been observed. To satisfy the needs of crop producers to obtain reliable yields under stressful environmental conditions as well as to quench consumer requests for healthy and versatile food products, several research efforts have been recently made to revisit benefits provided by often neglected traditional plant genetic resources. To detect specific phenotypic traits and to determine the role of collective action of genes in the traits' development among the wide array of abandoned genotypes available in gene banks around the world, a thorough and rapid assessment of diversity become a priority issue. Fortunately, this quest for new sources of plant diversity coupled with the development of new techniques of molecular biology that made possible deliberated utilization of landraces as a strategic starting material for both, breeding of cultivars adapted to stressful environments and suitable for production of functional foods. This review is intended to discuss the role of diversity of plant resources in the development of resistance to abiotic stresses. This issue will be illustrated using a case study of aluminium resistance among wild and cultivated plant species on the island of Madeira.

Soil acidity and aluminium stress

Soil acidity is a serious agricultural problem throughout many parts of the world, affecting as much as 40% of the world's arable land and up 70% of world's potentially arable land [1]. According to van Wambeke [2], acid soils occupy 1,455 million ha (11%) of world's land. The

global expanse of acid soils, with pH below 5.5 in their surface layers, is estimated to be 3,950 million ha, approximately 30% of total land in the world [2,3]. Soil acidity is influenced by edaphic, climatic and biological factors [4]. Soil primary materials, high rainfall and decaying organic matter that releases carbonic acid contribute greatly to soil acidification [5] and in the consequence limit crop productivity, plant distribution and colonisation of the ecosystems by plant species. Although the low fertility of acid soils is due to a combination of mineral toxicities of aluminium and manganese, and deficiencies of phosphorus, calcium, magnesium and molybdenum, aluminium ions are commonly considered as the major growth and yield-limiting factor on mineral soils with acid pH [6-8]. Under acidic conditions, monomeric Al ions are released to soil solution from soil minerals and polycationic, non-toxic Al complexes that exist at neutral pH. The ionic strength of acid soil solutions is lower than 5000 mM while the concentration of Al is lower than 5×10^{-3} cmol [9]. Once in soil solution, soluble Al ions can be taken up by roots and subsequently affect plant growth. The first visual symptom of Al toxicity is a drastic reduction of root growth [7,10]. The root system as a whole is affected, with many stubby lateral roots and absence of fine branching. Such roots are inefficient in absorbing nutrients and water [6], which typically leads to inhibition of biomass accumulation and decrease of grain yield and harvest index [11-13].

Plant species differ in their resistance to Al, whereas some are inherently more resistant than others [4]. Since during evolution, plants developed numerous mechanisms that allow survival in acid soils rich in available ionic Al [14,15]. As a result of selective pressure, inherently inter- and intra-species differences in response to toxic concentration of metals, including Al, are widely observed in the plant kingdom [16]. Speciation processes are particularly common in volcanic, acid soils where high amounts of exchangeable metals may originate both from the natural level of metal or the intensification of releasing processes into soil solution.

Several screening methods aimed at identification of the Al resistant genotypes have been developed [14,17]. The most popular physiological laboratory test that permits prediction of field resistance to Al involves measurements of root elongation under Al stress in hydroponic set-up. These measurements can be often facilitated by using eriochrome cyanine staining to visualize root regrowth after exposure to Al [10,14]. The importance of the screening towards this trait is also emphasized by the fact that aluminium and metal resistance is some instances may be correlated with other abiotic and biotic resistance traits [5,11,18].

Plant species of agricultural importance show a wide range of responses to Al toxicity. Among cereals, rye is considered to be the most Al resistant followed by oat, triticale, wheat and barley [14,19,20,21]. However, wheat is more resistant than maize and Brassicaceae species. Aluminium at 100 mM appears to be highly toxic to the majority of genotypes of wheat, while 50 and 25 mM Al are toxicity thresholds for maize and *Brassica* species, respectively [14,21-24]. Great differences in resistance to Al have been reported among genotypes originating from Brazil and South America where the intense selection pressure of acid soils with high aluminium supply resulted in the development of resistant wheat and maize cultivars [9,22,23]. These cultivars often serve as donors of genes controlling Al resistance in breeding programs throughout the world. Nevertheless, a pool of suitable gene sources is limited and identification of new Al-resistant genotypes could contribute to the diversification of selection efforts.

Crop Plant Diversity on the Island of Madeira

The Portuguese Archipelago of Madeira is located on the Atlantic Ocean, between latitudes 33°10' 32°20' N and longitudes 16°10' 17°20' W, 630 km west of the coast of North Africa, and consists of five islands: Madeira, Porto Santo, Deserta Grande, Deserta Pequena and Bugio. The main islands are Madeira with an area of 728 sq km (50 by 25 km), and Porto Santo with an area of 50 sq km. Madeira itself is the largest and highest of the islands, where Pico Ruivo (1861 m asl) and Pico Areeiro (1820 m asl) are the highest peaks.

The Island of Madeira that has a volcanic origin is characterised by the presence of high concentrations of several metals, including aluminium in both soil minerals and soil solution and prevailing acid soil pH [25,26]. One could expect that selective pressure of acid soils and widespread metal toxicity would predestine this island to be a good choice to search for new forms of metal resistant genotypes. To illustrate the role of natural and anthropogenic factors in the origin and development of Al resistance on Madeira we will briefly discuss three cases of domesticated and wild plant species, including wheat, maize and *Sinapidendron*.

Wheat

Cultivation of wheat (*Triticum* ssp.) on the Archipelago of Madeira has begun with island colonisation, when the first cultivars have been introduced from the Portuguese midland [27]. High wheat diversity on Madeira has been established as a result of introduction of cultivars brought from various phytogeographical locations around the world as early as in the fifteenth century [27,28]. At least three major wheat introductions in the Archipelago of Madeira were reported, including importation events from the Portuguese mainland during the fifteenth century [27], from Azores and Canary Archipelagos and North of Africa in the sixteenth century [29] and from the North and South Europe during the seventeenth century [29,30]. A preliminary evaluation of wheat resources collected and preserved at the ISOPlexis Germplasm Bank revealed that they belong to *T. aestivum* L. subsp. *aestivum* L., *T. aestivum* L. subsp. *compactum* Host, *T. turgidum* L. subsp. *durum* (Desf.) Husn, *T.turgidum* L. subsp. *turgidum* (Desf.) Husn [28]. The evaluation of morphological, biochemical and molecular traits of wheat accessions allowed us to classify them into 16 varieties and 42 landraces [dos Santos, unpublished data]. Surprisingly high diversity of wheat resources existing presently on a fairly small island resulted from the crop management practices that have been continued by generations of local farmers, who frequently operated on small plots located on terraces plough in on steep slopes of remote and isolated mountain valleys. The farmers have been using for decades their own stocks of wheat seeds that supposedly were introduced to the island centuries ago.

Maize

Maize (*Zea mays* L.) was introduced to the Island of Madeira in 1760 [27] more than 200 years after the first maize introductions in Europe [31]. In 1847, with the improvement of the irrigation facilities on the island this crop was adopted for human food and animal feed. First introductions of maize germplasm occurred in the eighteenth century from the Archipelago of Azores, where maize was already an established crop [32]. Subsequent introductions took place from Azores, Canary Islands, and the Portugal mainland and possibly from Africa. Since then farmers have been using the local market to obtain seeding material for their own production and the external

introductions of maize germplasm seem to have been sporadic. The first study aimed at the evaluation of maize diversity on the island has shown that the existing resources could be classified into four landrace's groups, which one apparently consists of several cultivated forms and genotypes. These landraces have some resemblance to the Caribbean eight rows landrace, which they have been apparently originated from [33].

Sinapidendron

Sinapidendron is an endemic genus of native plants inhabiting the Archipelago of Madeira [34], which represent wild relatives of the cultivated *Brassica* crops. This genus is represented by five species of small perennial shrubs, while four of them can be found on the Island of Madeira, including *S. gymnocalyx* (Lowe) Rustan; *S. angustifolium* (DC) Lowe; *S. frutescens* Lowe; *S. ruprestre* Lowe, and one *S. semprevivifolium* Menezes on the Deserta Grande Island [Short, 1994]. *Sinapidendron* species can be differentiated by their morphology, rarity and ecosystem distribution. *S. gymnocalyx* is a glabrous shrub, common in the cliffs of the north coast from sea level to 500 m asl, sporadically found at altitudes up to 1000 m asl. *S. angustifolium* is a glabrous slender shrub with acute and linear leaves. Its distribution is limited to the south coast of Madeira on the cliffs and near the sea slopes from the sea level to 200 m asl. *S. frutescens* is a slender lower adpressed-pubescent shrub with elliptic to elliptic-oblanceolate leaves [34,35). It can be found in cliffs and slopes along the north coast and in ravines of central peaks of the island up to 1800 m asl. *S. ruprestre* is a perennial herb with woody below and coarse oblong to ovate leaves irregularly dentate, that can be found in ravines and mountains of the northern Madeira from 850 to 1500 m asl. Some of them, such as S. *gymnocalyx* are traditionally used by farmers as forage for cattle and cultivated on the borders of agricultural fields.

As indicated above, both cultivated crops (wheat and maize) resulted from multiple introductions during past several centuries from different geographical regions [27,28,30,33], while *Sinapidendron* species currently present on the island have a narrow diversity resulted from isolated colonization events, one event according to Vargas [36], presumably from the Mediterranean basin, followed by adaptation to certain ecological niches.

We hypothesise that the cultivated species and wild relatives of crop species differ greatly in their ability to withstand Al stress due to unique management practices on the island, i.e. preservation of local seed stocks [29] and initial plant diversity. It is reasonable to claim that the initial artificially introduced variability could have been maintained throughout centuries and even increased by farmer's selection. Human intervention has also considerably contributed to the present distribution of cultivars on the island. In the past, plots with predominantly stringent Al stress conditions were instinctively seeded to the cultivars that would guarantee reasonable yield, while the under-performers were either discarded or planted on plots of less stressful conditions [26,35,37]. Cultivars of high initial resistance subjected to stringent environmental conditions over extended period of time acclimated to the specific edaphic conditions existing on a given plot and they enhanced further their ability to withstand Al stress.

AL Tolerance among Crop Plant Species on the Island of Madeira

At the ISOPlexis Germplasm Bank of the University of Madeira we have conducted

comprehensive studies aimed at an assessment of response of cultivated and wild species to aluminium toxicity and several soil edaphic conditions, including pH, aluminium content and organic matter. Soil samples of top 20 cm of soil were collected at a range of locations around the Island of Madeira. At 121 sites we have also collected seeds of 59 cultivars of wheat (*Triticum* ssp.), 46 cultivars of corn (*Zea mays*) and 16 populations of *Sinapidendron* ssp. The soil pH was measured according to the Forster method [38]. The exchangeable aluminium and organic carbon content of soil samples were analysed according to the modified hydroxylamine acid method [39] and the modified Walkley-Black method [40], respectively. Wheat and corn seeds were grown hydroponically, while seeds of *Sinapidendron* were grown in MS solid medium as described elsewhere [35]. Seedlings have been exposed for 72 h to aluminium at concentrations ranging from 25 to 200 iM according to methodology described earlier [26,41]. After Al treatment, plant roots were stained with eriochrome cyanine R_{250} (Sigma) to visualise root survival and regrowth [10,14]. Root tips of plants that were able to continue growth after exposure to Al remained white, while roots with irreversibly damaged apical meristems were dark purple what indicated the absence of regrowth even after transfer to Al free medium. The obtained results were used to assess diversity of the cultivated and wild species in relation to aluminium QTL and to identify groups of interesting genotypes with different ranges of response to the presence of aluminium for further research and breeding programs.

Topsoil samples collected from the fields grown to wheat and corn or inhabited by *Sinapidendron* populations were analysed for their acidity. The pH of topsoil sampled from the sites across the island was ranging from 3.83 to 7.97, with a mean soil pHKCl of 5.01. Normal-like frequency distribution of soil pH was noticeably shifted towards acid values and 60% of the plots exhibited pH below 5.0. Only 24.0% of the plots had pH above 5.5. However, among the *Sinapidendron* populations 81.3% of the soil samples had pH above 5.5, whereas for wheat and maize these values were 15.3 and 15.2%, respectively [26,35,37]. Low mean pH of the Madeiran soils was accompanied by high amounts of exchangeable Al. On average, 0.79 cmol of ionic Al per kg of soil was found in the analysed topsoils. Fifty one per cent of the sampled soils showed levels of exchangeable Al exceeding 0.9 $cmol.kg^{-1}$ of soil. A negative correlation between soil ionic aluminium content and soil pH was found. Topsoil samples exceeding 0.9 cmol of aluminium per kg of soil reach 61.7 % in the wheat populations, 53.3 % for maize and only 20.0% for *Sinapidendron*. Interestingly, soil aluminium content increased along with elevation and the majority of populations of *Sinapidendron* were found at elevations below 200 msl. We have also measured content of organic matter (OM) in all soils. On average the tested soils contained 3.2% of OM, while only two tested sites contained extremely high amounts ranging about 7.5%. Fifty per cent of topsoils of *Sinapidendron* populations have OM above 3.2%, whereas majority of topsoils from wheat (52.8%) and maize (51.0%) shows values below this level [26,35,37].

We expected that edaphic parameters such as soil pH, ionic Al or organic matter and observed variation in their values could contribute to the development of Al resistance among both crop and wild species found on the island. To verify our hypothesis we have evaluated performance of the hydroponically grown plants under Al stress using the eriochrome cyanine test. To achieve the separation between cultivars or populations in response to Al we had to subject wheat and corn seedlings to the very stringent stress conditions of 72 h exposure at 100 and 200 iM Al or 25 and 50 iM Al in the case of *Sinapidendron*. Indeed, we found that the

degree of response of the Madeiran genotypes to Al stress imposed in nutrient solution correlated with the amount of ionic Al in soils, regardless of the plant species tested [17,26,35,37]. Relationship between soil OM and level of Al resistance was more complicated. In corn enhanced resistance was associated with higher amounts of organic matter found in soil. However, in the wild species *Sinapindendron*, a negative correlation was found when all plots were taken into consideration. Surprisingly, no correlation between OM and Al resistance has been found in wheat [26,35,37].

We were unable to identify an obvious pattern of relationship between Al resistance of the tested species and plot altitude. The only exception was *Sinapiadendron* were toxic aluminium ions presented in soil solution seem to be a factor limiting distribution of *Sinapidendron* at lower elevations (below 200 m asl), with the exemption of *Sinapidendron frutescens*, found in mountainous region of the Island of Madeira.

Enhanced resistance to aluminium appeared to be a common phenomenon among the Madeiran plant species, especially among cultivated species [17,26,35,37]. Our previous studies indicated that some of the Madeiran wheat cultivars are more Al resistant than the worldwide-accepted standards of Al resistance [26,41]. At the same time, the development of plant resistance to the presence of aluminium seems to be a less frequent phenomenon among *Sinapidendron* species and can be determined by the features of island colonization by these species [36]. The initial genetic variability of plant populations is a critical parameter for the successful development of plant resistance to the soil metals [11].

Beside of the anthropogenic factors, specific soil conditions present on the island had profound effects on the current distribution of both cultivated and the wild species. As very acid andosols dominate on the island [42], low mean pH of the Madeiran soils resulted in the enhanced release of high amounts of exchangeable Al to soil solution. Due to economical reasons liming, a commonly used practice of controlling soil acidity, is not a viable option for the Madeiran agriculture. Moreover, efficacy of this technique in soil amelioration has been frequently questioned [4]. Additionally, we observed that the aluminium content in soil increases with the altitude, most likely because of lower buffering capacity of soils at earlier stages of development and lower content of organic matter. Our data seem to support a hypothesis that the Al content in soil solution is the prime environmental factor involved in development of Al resistant genotypes and it could be a major factor determining distribution of cultivars/ecotypes across the island. The most Al resistant genotypes of the cultivated species as evaluated by the eriochrome cyanine test were collected from plots containing high amounts of Al^{3+}. However, in the case of *Sinapidendron*, were the evolution of aluminium resistance seems to be an sporadic event, exchangeable aluminium is a factor limiting island colonization to the sites exhibiting less stressful conditions.

Al toxicity in soils is often affected by organic matter content. In the presence of organic matter, the toxicity of Al in soils is considerably reduced presumably due to the detoxifying effect of organic acids or via the formation of Al-organic complexes [43,44]. Our data indicate that Al resistance was somewhat associated with the levels of organic matter in soil. This relationship was observable only in corn while less evident in *Sinapidendron* and not present in wheat at all. The differences between two cultivated species in response to OM might be due to different cultivation practices applied for centuries on the island. As a rule, corn plots had received much higher levels of organic fertilizers (manure, plant residues) than wheat.

This could lead to a preferential accumulation of OM in plots seeded to corn, which contained high levels of Al. Thus, one could argue that it is possible that some toxic Al-complexes (such as polymeric Al_{13} tridecamer) [15] could prevail in cultivated corn plots and these conditions might favour selection towards Al resistance. Analysis of 16 populations of *Sinapidendron* may shed some additional light on a function of OM in acquiring of Al resistance by wild species on the Island of Madeira. It is apparent from our data that populations grown on sites with extremely high levels of OM were more susceptible to Al as was revealed by a test in hydroponic solution. It could be explained by the fact that the abundance of OM could reduce toxic effects of Al, and thus, these particular sites were inhabited by less Al resistant populations. We incline to suggest that organic matter content played a secondary role in the development of Al resistance and cultivar distribution across the island, and perhaps it only reduced toxic effects of Al to wild species.

Breeding of Wheat for Aluminium Tolerance and Search for Physiological and Molecular Mechanisms of Al Tolerance

We have identified several wheat genotypes among the Madeiran germplasm that differ drastically in their ability to tolerate the aluminium stress [26,35,37]. We have developed pure lines of several of these genotypes that served as a model system used to identify and characterize some of the physiological and molecular mechanisms involved in the stress response. Out of over 50 cultivars originally screened towards the Al resistance, seven cultivars representing a whole spectrum of responses to Al stress found on the island were chosen, including the Al resistant, semi-resistant and sensitive genotypes. Two parallel approaches to develop a model material suitable for physiological and molecular studies were used. In the first approach, five genotypes were selected either for resistance or sensitivity until the F_3 generation. In the second approach, double haploid lines of four genotypes of different Al resistance were developed. In both approaches taken, Al resistance of the genotypes obtained in each generation was comprehensively assessed using a battery of physiological tests including root elongation eriochrome cyanine screening, aluminium uptake in roots and accumulation of callose in root tips. Additionally, to get an ultimate proof of plant performance under Al stress total biomass production was measured.

Exudation of malic acid to the rhizosphere that has been recently recognized as the main factor contributing to the Al resistance in wheat [45], is supposedly controlled by only one major *ALMT1* gene [46-49]. However, conflicting arguments have been presented by some other researchers [21,50,51] and our data appear to support the concept of a polygenic control of Al resistance in the Madeiran wheats. The preliminary results of the thorough physiological tests and the segregation ratios seem to indicate that more than one gene is involved in response to the aluminium stress and that Al resistance is controlled by more than one mechanism. These conclusions are supported by the variation in the resistance levels as measured using the eriochrome cyanine test and supported by root elongation results in the three obtained generations. Among the genotypes initially classified as Al resistant we have observed a further enhancement of resistance in the F_1 generation, a sharp decrease in F_2, and subsequent increase of resistance in the F_3 generation. On the other hand, in the sensitive cultivars a decrease of Al resistance in the F_1 and F_2 generations followed by a slight increase in the F_3 generation was observed. Interestingly, using the sensitive callose deposition test we were unable to detect

increase in resistance in the F_3 generation of the sensitive genotype, which might further indicate that different mechanisms could be responsible for the expression of Al resistance in the third generation. Currently we are continuing process of selection and evaluation of these genotypes until the fifth generation. Concurrently, detailed biochemical characterizations of the parental generations, including enzyme kinetics, mRNA expression studies, root and leaf protein expression under Al stress assessed by the 2D electrophoresis are being conducted. Comparison of these results with the F_3 and double haploids data is expected to give us an insight in to the genetics of Al resistance and hopefully it will contribute to the identification of mechanisms involved in responses to Al stress among the Madeiran wheat cultivars. Our endeavours will be also complemented by screening of the Madeiran germplasm for genes involved in Al resistance using the micro arrays technology [52].

Conclusions

The Madeiran cultivars of wheat and maize as well as wild populations of *Sinapidendron* exhibit a wide range of responses to Al stress. Several genotypes possessing enhanced resistance to Al that have been identified on the island could serve as a source of genes controlling Al resistance for the classical or marker assisted breeding programs [17,26,35,37]. We hypothesise that the diversity of responses to aluminium is associated with different QTL phenotypes and could result from a possible involvement of both environmental and anthropogenic factors in the diversification processes. Anthropogenic factors, i.e. the introductions of germplasm from the overseas that occurred over last five centuries, selection of the best performing cultivars by local farmers and agricultural practices dominating on the island have significantly contributed to the development of multiple responses to aluminium among wheat and maize landraces. On the other hand, development of Al resistance among ecotypes of *Sinapidendron* entirely depended on natural processes of speciation and colonization. Studies aimed at elucidation of the contribution of different genes to the QTL phenotypes from the Island of Madeira are warranted.

Acknowledgements

Portuguese Foundation for Science and Technology (FCT, Fundação para a Ciência e Tecnologia) has sponsored this work, through the Centre of Macaronesian Studies (CEM) and the project POCTI/ NO 41040/ AGR/ 2001. The authors are grateful to Professor Janusz Zimny from the Plant Breeding and Acclimatization Institute, Radzikow, Poland for the development of double haploid lines of wheat.

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