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Arabidopsis as a Model System to Study Chilling Tolerance Mechanisms in Plants

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ABSTRACT

Many plants of tropical and subtropical origin, including a large number of economically important crops, such as tomato, rice, cotton, cucumber and maize, are severely damaged when exposed to temperatures between 2 and 15°C (chilling temperatures). The symptoms of these chilling injuries include cessation of growth, wilting, chlorosis, and necrosis. In contrast with chilling-sensitive species, the cruciferous plant *Arabidopsis thaliana* is chilling tolerant, and is able to grow to maturity even at a low temperature of 4°C. Therefore, at the genetic level, Arabidopsis may provide a useful model plant system for the identification of chilling-tolerance traits. Taking a mutational approach several ethyl methanesulphonate (EMS) and T-DNA insertion chilling-sensitive mutants have been identified that show wild-type phenotypes when grown at normal temperatures, but are severely damaged following transfer to low temperatures. These mutants provide valuable genetic sources for the identification of structural or regulatory genes that are crucial for plant survival at chilling temperatures. Furthermore, it has been reported that a number of mutations at several genetic loci involved in fatty acid biosynthesis (*fab1*) and fatty acid desaturation (*fad2*, *fad5* and *fad6*) resulted in reduced-growth and chlorosis phenotypes at low temperatures, thus providing direct evidence for the contribution of lipid polyunsaturation to low-temperature fitness. Arabidopsis has also proven to be an efficient model system for the identification of major biochemical mechanisms involved in protection of the photosynthesis system from photooxidative damage following exposure to excess light energy at low temperatures. DNA microarray studies have revealed new insights into the complex network of transcriptional regulation at low temperatures and the possible interrelationships between cold-regulated gene expression and acquisition of chilling tolerance but this work is just beginning. At last, recently, Arabidopsis is also being used as a main model plant system to study possible genetic linkages between the programmed cell death (PCD) mechanism and development of necrotic lesions following exposure to biotic and abiotic stresses, including chilling. Overall, it is concluded that Arabidopsis can potentially be an ideal model system for basic studies on chilling stress and for identification of key components of chilling-tolerance traits in plants.

Keywords: chilling-sensitive mutants, fatty acid desaturase, low temperature
Abbreviations: CHS, chilling sensitive; **COR,** cold-regulated; **ELIPs**, early light-inducible proteins; **EMS**, ethyl methanesulphonate; **PCD**, programmed cell death; **PG**, phosphatidylglycerol

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INTRODUCTION

Low temperature is an important environmental factor, which greatly influences the growth, development, survival, and geographical distribution of plants (Levitt 1980). Whereas most plant species from temperate regions can acclimatize to cold and can survive exposures to deep freezing temperatures, plants of tropical and sub-tropical origin are severely injured or killed when exposed to low non-freezing temperatures between 0 and 15°C (Lyons 1973; Lynch 1990; Wang 1990). Exposure of chilling-sensitive plants to low temperatures leads to various physiological alterations, such as increased electrolyte leakage from the cytosol through the cell membrane, and decreases in photosynthetic capacity and respiration rates. The symptoms of chilling injuries include cessation of growth, wilting, chlorosis, necrosis, and eventually, plant death (Lyons 1973; Graham and Patterson 1982; Maruyama *et al*. 1990; Allen and Ort 2001). In addition to its adverse effects on plant growth and development, chilling sensitivity also imposes major limitations on the postharvest storage and handling of fruit and vegetables, since it necessitates storage at relatively high temperatures, which enhance deterioration and spoilage (Paull 1990; Kader 2002).

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Invited Review

In contrast to our knowledge of plant responses to other abiotic stresses, such as freezing, drought, salinity, and heat, little is yet known regarding the molecular basis of chilling tolerance, or of the signal transduction networks involved in its acquisition. In previous reviews, the occurrence of chilling damage was attributed mainly to general disruption or dysfunction of cellular metabolic and physiological processes (Lyons 1973; Graham and Patterson 1982; Markhart 1986). Nevertheless, several lines of evidence suggest that an important primary event in the occurrence of chilling injury is an alteration in the physical state of the cellular membranes, which leads to their reduced selective permeability and results in solute leakage from the cells (Lyons 1973; Markhart 1986; Nishida and Murata 1996). The importance of the content and composition of membrane lipids for plant responsiveness to chilling has been demonstrated in transgenic plants, in which increasing the levels of lipid polyunsaturation increased membrane fluidity and tolerance to chilling (Murata *et al*. 1992; Kodama *et al*. 1994). Another factor that affects susceptibility to chilling is the status of the cellular antioxidant defensive system that is required to metabolize the reactive oxygen intermediates responsible for peroxidation of fatty acids and disruption of membrane function. Indeed, overexpression of antioxidant defensive genes, such as ascorbate peroxidase (APX), superoxide dismutase (SOD) and catalase (CAT) increased chilling tolerance (Van Breusegem *et al*. 1999; Payton *et al*. 2001), whereas repression of CAT gene expression reduced it (Kerdnaimongkol and Woodson 1999). Finally, several reports have suggested that various stress genes, usually related to other types of stress responses, may also contribute to the acquisition of chilling tolerance. For instance, it has been suggested that certain heat shock proteins (HSPs) (Sabehat *et al*. 1998; Sung *et al*. 2001) and dehydrin genes (Ismail *et al*. 1999) may also be involved in imparting chilling tolerance to plants.

In the present review, we will summarize data obtained from studies on chilling tolerance and susceptibility in Arabidopsis, and will suggest how the adoption of Arabidopsis as a plant model system could improve our basic understanding regarding the molecular and biochemical nature of chilling tolerance. Overall, Arabidopsis' many advantages as a plant model system and its chilling tolerance make it an excellent study subject for the identification of major genetic traits important for low temperature survival of plants.

USING ARABIDOPSIS AS A GENETIC SOURCE FOR THE IDENTIFICATION OF CHILLING TOLERANCE TRAITS

In the past, most of the research on plant responses to chilling stress focused on determining the physiology and biochemical processes that contributed to the development of chilling injuries, or on the development of horticultural means to ameliorate them (Lyons 1973; Graham and Patterson 1982; Lynch 1990; Wang 1993). However, from a genetic perspective, it may actually be much more efficient to obtain biological information regarding the nature of chilling tolerance by studying the molecular and biochemical mechanisms operating in temperate-origin plants that are able to grow and survive at low temperatures rather than studying the processes that lead to damage in chilling-sensitive species. In the case of Arabidopsis in contrast to nearly all chilling sensitive species, it can be relatively easy to screen large mutagenized populations for individuals sensitive to chilling conditions resulting from a loss-of-function mutation in an important gene. To generate a gain-of-function mutation in a chilling sensitive species that enhances chilling tolerance is a far less likely probabilistic event and likely to be much more difficult. For populations with insertional mutations, the compromised genetic locus can be readily obtained, the gene isolated, sequenced and its function potentially identified. Additional follow up complementation studies can confirm the linkage of a mutant gene with the ability of the plant to tolerate chilling. For point mutations, identifying the affected locus is more involved requiring linkage map positioning before the characterization and biological testing can be undertaken.

It is believed that chilling-sensitive species evolved in warm tropical regions in which there was no selection pressure favoring growth at low temperatures. However, the dispersal of plants to cooler climates would have necessitated the acquisition of chilling tolerance. Indeed, trees and shrubs in temperate regions are able to grow at low, chilling temperatures and become dormant during the coldest portion of the winter when temperatures are below 0°C (Levitt 1980). Many herbaceous annual species such as Arabidopsis normally overwinter in their vegetative state, survive both chilling and freezing conditions, and switch to reproductive production during the spring, when temperatures rise. Thus, herbaceous annual species can be very resistant to chilling, and continue their growth and development, albeit more slowly, at low temperatures. Indeed, we recently have shown that Arabidopsis can complete its entire life cycle and produce fertile seeds even when sown and grown under continuous chilling temperatures of 6°C (Hasdai *et al*. 2006) and 4°C (Porat and Guy, unpublished). Therefore, truly chilling-resistant plants, such as Arabidopsis, may provide valuable genetic sources for the identification of chilling-tolerant traits which, in the future, may be possible to incurporate into horticulturally important chilling-sensitive plants (Tokuhisa and Browse 1999). It should be noted that although some genetic variations have been observed in the responses of different Arabidopsis ecotypes (genotypes collected from distinct locations) to chilling and freezing temperatures (Klotke *et al*. 2004; Hasdai *et al*. 2006), there is, so far, no clear association between the flowering habit and the requirement for vernalization and the geographical distribution of the various ecotypes (Pigliucci 1998).

In the following sections, we will summarize some of the major findings regarding the molecular and biochemical mechanisms thought to be involved in conferring chilling tolerance on Arabidopsis, with special emphasis on the identification of chilling-sensitive (chs) mutants, genes involved in modifications of membrane lipids composition, protection of photosynthesis, programmed cell death and transcriptional regulation at low temperatures.

Chilling-sensitive mutants

In order to study the molecular basis of chilling tolerance in Arabidopsis, EMS-mutagenized M_2 populations of Arabidopsis were grown at 22°C for 2 weeks after which they were transferred to 10 or 15°C and were screened for the appearance of chilling-sensitive mutants (Schneider *et al*. 1995a). Of about 20,000 M_2 plants examined, 21 mutants were identified that appeared normal at 22°C but developed chlorosis or necrosis when shifted to lower temperatures (Schneider *et al*. 1995a). Chilling-sensitive mutants were placed into four different phenotypic classes: class 1 mutants (*chs1*, *chs2*, and *chs3*) turned yellow, wilted and died; class 2 mutants (*chs4*) showed similar responses, but only the mature leaves become necrotic; class 3 mutants (*chs5-6*) developed yellow chlorotic patches but continued to grow and develop at low temperatures; and in class 4 (*chs7-15*) only part of the leaf near the rosette turned yellow (Schneider *et al*. 1995a; **Fig. 1**). Crosses among mutants in different phenotypic classes showed that those in the first three classes were found only in a small number of loci (Schneider *et al*. 1995a). Furthermore, all the *chs* mutants were recessive except for *chs2*, which was dominant (Schneider *et al*. 1995a).

Detailed and extensive characterization of the *chs1* mutant revealed that it was sensitive to temperatures below 18°C, and that exposure to low temperatures resulted in defects in chloroplast maintenance and integrity, including disruption of chloroplast protein accumulation and altered steryl-ester metabolism (Hugly *et al*. 1990; Patterson *et al*. 1993; Schneider *et al*. 1995b). Furthermore, it was found that only the leaf tissues of *chs1* plants were injured at low

Fig. 1 Phenotypes of Arabidopsis wild type (Colombia ecotype) and *chs1*, *chs2*, *chs3*, *chs4*, *chs5* and *chs6* mutants. Photographs were taken after 2 weeks of growth at 22°C followed by 2 weeks at 14°C.

temperatures whereas germination, root and callus growth were unaffected by chilling. All these findings suggest that the function of the *chs1* gene product may be specifically required to maintain chloroplast function at low temperatures. Overall, the *chs1* mutant was found to be extremely sensitive to low temperatures, and after 3 days at 13°C, the plants become irreversibly injured and could not be rescued upon returning them to normal temperatures (Schneider *et al*. 1995b). Interestingly, it was also noted that the *chs1* mutants were much more sensitive in terms of leaf yellowing and time till the initiation of wilting at 15°C than at 5°C (Schneider *et al*. 1995b).

Recent transcriptome profiling studies among approximately 8,000 Arabidopsis genes that compared gene expression patterns in wild-type plants with those in 12 chilling-sensitive mutants showed that the expression of more than 1,000 genes in normal plants was unaffected by chilling at 13°C but was affected by at least 2-fold in class 1 *chs* mutants (Provart *et al*. 2003; Zhu and Provart 2003). In the light of these microarray expression data it was suggested that the functions of the mutated *chs1*, *chs2*, and *chs3* genes might be to prevent widespread chilling damage effects on transcriptional regulation (Provart *et al*. 2003). It was further observed that the profiles of gene expression of the various class 1 *chs* mutants (*chs1*, *chs2*, and *chs3*) at 13°C were very similar to each other, which supports the idea that the products of these genes might perform related biological functions. In the future, identification of the gene products of class 1 *chs* mutants by means of mapping and chromosome walking technologies will certainly provide important insights into the molecular basis of at least some major factors that are crucial for plant survival at chilling temperatures.

Among the 21 *chs* mutants identified (Schneider *et al*. 1995a), the only gene that has been cloned so far is *chs5*; it belongs to class 3 of *chs* mutants, which become chlorotic at low temperatures but otherwise continue to grow and develop normally. Genetic and sequence analysis demonstrated that the *chs5* mutation occurred in the coding region of 1-deoxy-D-xylulose 5-phosphate synthase (DXS), an enzyme belonging to the non-mevalonate pathway localized in the chloroplast (Araki *et al*. 2000). DXS functions in the synthesis of isoprenoid compounds like carotenoids, xanthophylls, sterols and isopentynyl chains of cytokinins and chlorophylls. Once again, as in the case of the *chs1* mutation, it seems that among the diverse components of cellular machinery, the chloroplast is especially vulnerable to low chilling temperatures.

In a different study, Tokuhisa *et al*. (1997) identified additional *Arabidopsis* EMS and T-DNA insertion chilling-sensitive mutants that were indistinguishable from wild-type plants when grown at 22°C, but that exhibited visible chilling symptoms after a long incubation period of 42 days at an extreme low temperature of 5°C. Under these selective growth conditions, the chilling symptoms that were identified included chlorosis, reduced and impaired growth (small stature, reduced leaf growth, high anthocyanin content, and distorted leaf morphology), necrosis, and death. In the light of this diversity of phenotypes, it was concluded that chilling tolerance in Arabidopsis involves diverse processes, including organelle biogenesis, cell metabolism, and organ development processes (Tokuhisa *et al*. 1997). Thus, two independent populations of chilling-sensitive mutants have been identified so far in Arabidopsis: the mutants described by Somerville and coworkers (Schneider *et al*. 1995a) were already apparent after a short exposure (7 days) to mild temperatures (10-15°C), whereas the mutants described by Browse and coworkers (Tokuhisa *et al*. 1997) were apparent only after a much longer incubation period of 6 weeks at a very low temperature of 5°C (Schneider *et al*. 1995a; Tokuhisa *et al*. 1997). The first type of mutants, especially class 1 *chs* mutants, most likely encode proteins crucial for basic aspects of cell survival at chilling temperatures, whereas the second type of mutants probably involves governing particular mechanisms of adaptation to low temperatures of older cells and organs or are characteristic of older cells. One of the latter T-DNA-tagged mutants, *paleface1* (*pfc1*), that becomes chlorotic during growth at 5°C, was cloned and encodes a specific 16S rRNA methylase, which is required for maintenance of a particular step in pre-RNA processing in the chloroplasts, and which is apparently sensitive to low temperatures (Tokuhisa *et al*. 1998).

Mutants in fatty acid composition

Several studies have confirmed that there is a tight correlation between chilling sensitivity and the fatty acid composition of phosphatidylglycerol (PG) in chloroplast membranes (Murata *et al*. 1982; Nishida and Murata 1996). Chloroplast PG always contains 16:0 or 16:1 fatty acids in the C2 position of *sn*-glycerol-3-phosphate. However, in

chilling-resistant plants the unsaturated fatty acid, oleic acid (18:1) tends to be found at the C1 position of *sn*-glycerol-3-phosphate, whereas in chilling-sensitive plants the saturated fatty acid, palmitic acid (16:0) tends to be present. These differences largely result from substrate selectivity of the enzyme glycerol-3-phosphate-acyltransferase. Overexpression of this gene from chilling resistant Arabidopsis plants into tobacco reduced the saturated PG levels and appears to enhance chilling tolerance, whereas overexpression of presumably the same gene ortholog from chilling-sensitive squash plants increased the content of saturated PG and reduced chilling tolerance (Murata *et al*. 1992). Furthermore, alteration of Arabidopsis PG chloroplast lipids by overexpression of the *E. coli* glycerol-3-phosphate-acyltransferase (*plsB*) gene which is selective for the incorporation of saturated palmitic acid (16:0) instead of oleic acid (18:1) resulted in increased amounts of saturated fatty acids in the plastids, and consequently reduced chilling tolerance in Arabidopsis. These chilling-susceptibility symptoms became apparent, as enhanced wilting, browning and necrosis of older rosette leaves, after 7 days of exposure to 4°C (Wolter *et al*. 1992).

Another mutant strain of Arabidopsis, fatty acid biosynthesis1 (*fab1*), contains increased proportions of saturated palmitic acid (16:0) in its cellular lipids because of a defect in 3-ketoacyl-acyl carrier protein synthase II, the enzyme responsible for the elongation of 16:0 to 18:0. As a consequent, the saturated PG content of Arabidopsis increased from about 9% in wild-type plants to 43% in *fab1* mutants, which resulted in enhanced chilling sensitivity, as indicated by leaf chlorosis and decreased photosynthetic capacity following long-term exposure of more than 7 days at 2°C (Wu and Browse 1995; Wu *et al*. 1997). Nevertheless, it should be noted that in spite of the high levels of saturated fatty acids obtained in *fab1* mutants, which were similar to those present in many chilling-sensitive species such as cucumber and tobacco, the *fab1* plants were still able to survive short-term exposures to chilling (up to 7 days at 2°C under constant illumination); such conditions usually lead to the death for many chilling-sensitive species. Therefore, it was concluded that chilling tolerance in Arabidopsis must be governed by a variety of traits, and that the control of the levels of lipid desaturation is only one major contributing factor among a rather much more complex process (Wu and Browse 1995; Wu *et al*. 1997).

Arabidopsis has proven to be an effective model system for studying the biochemical pathways involved in lipid metabolism, and especially fatty acid desaturation in plants, by screening for mutants with alterations in their membrane lipid composition (Somerville and Browse 1991). Overall, seven Arabidopsis mutants (*fad2*, *fad3*, *fad4*, *fad5*, *fad6*, *fad7*, *fad8*) with deficiencies in fatty acid desaturation have been identified, and each mutation affects a unique gene encoding a desaturase activity directed towards a specific class of acyl groups at a specific position on the acyl chain. Four of these mutations – *fad3*, *fad4*, *fad7* and *fad8* – had no direct effects on their own on the ability of the plants to develop at 5°C, whereas the other three – *fad2*, *fad5* and *fad6* – appeared to be chilling sensitive.

The *fad2* mutant of Arabidopsis is deficient in activity of the microsomal 18:1 desaturase responsible for the production of polyunsaturated lipids (Miquel *et al*. 1993; Okuley *et al*. 1994). At 22°C the *fad2* mutants showed growth characteristics that were similar to those of wild-type plants but, in contrast, at 12°C they failed to exhibit stem elongation during reproductive growth, and after transfer to 6°C, the rosette leaves became necrotic and gradually died. In the light of these results, it was concluded that polyunsaturated fatty acids are essential for maintaining cellular function and plant viability at low temperatures (Miquel *et al*. 1993).

The *fad5* mutant is deficient in the activity of a chloroplast ω-9 fatty acid desaturase, and it accumulates high levels of palmitic acid (16:0), whereas the *fad6* mutant is deficient in the activity of the chloroplast ω-6 fatty acid desaturase, and it accumulates high levels of 16:1 and 18:1 fatty acids (Hugly and Somerville 1992). The phenotypes of the *fad5* and *fad6* mutants were indistinguishable from wild-type plants when grown at 22°C, but the leaf tissues of the mutants that developed during growth at 5°C were chlorotic, whereas wild type leaves were not. This indicates that chloroplast lipid polyunsaturation is essential for low-temperature fitness (Hugly and Somerville 1992).

It is known that chloroplast membranes contain very high proportions of trienoic fatty acids (polyunsaturated fatty acids that have three *cis* double bonds). For example, α-linolenic (18:3) and hexadectarienoic (16:3) acids typically account for approximately two-thirds of all the thylakoid membrane fatty acids (Routaboul *et al*. 2000). In Arabidopsis, three gene products – *FAD3*, *FAD7* and *FAD8* – mediate the synthesis of trienoic fatty acids from 18:2 and 16:2. The *FAD3* gene product is an endoplasmic reticulum desaturase, and the *FAD7* and *FAD8* genes encode two chloroplast isozymes that recognize as a substrate either 18:2 or 16:2 attached to any of the chloroplast lipids (Somerville and Browse 1991; Hugly and Somerville 1992). A mutation in any one of these genes on its own results in only a marginal reduction of the trienoic fatty acids content; but leaves of the double mutant *fad7/fad8* have a trienoic fatty acids content of 17%; and the triple mutant *fad3/fad7/fad8* has essentially no 18:3 or 16:3 fatty acids in the thylakoid or any other membrane of the cell (Routaboul *et al*. 2000). It was found that the *fad3/fad7/fad8* triple mutant had morphological, growth, and developmental characteristics that were very similar to wild-type plants when grown at 25°C. Thus, presence of trienoic fatty acids is not absolutely necessary for growth and photosynthesis in Arabidopsis. However, following transfer of the triple-mutant plants to a chilling temperature of 4°C, it was found that photosynthetic processes were only subtly affected in the short term, but prolonged growth at 4°C, for several weeks, resulted in gradual deterioration of photosynthetic functions. In sum, it was concluded that in Arabidopsis trienoic fatty acids are important to ensure correct biogenesis and maintenance of chloroplasts during growth at low temperatures (Routaboul *et al*. 2000).

Finally, it was also demonstrated in Arabidopsis that it is not only fatty acid biosynthesis and desaturation genes that affect chilling tolerance: genes whose products affect membrane lipid asymmetry and fluidity also do so. For example, down-regulation of *ALA1*, an aminophospholipid translocase, that increases membrane fluidity by generating asymmetry through lipid flipping, resulted in a decrease in chilling tolerance, as indicated by reduced growth and yield at low temperatures of 8-12°C (Gomes *et al*. 2000).

Protection of photosynthesis at low temperatures

When light energy absorbed by plants becomes excessive relative to the capacity of photosynthesis, the xanthophyll, violaxanthin, is reversibly deepoxidized to zeaxanthin (violaxanthin cycle) (Havaux and Niyogi 1999). The *npq1* mutant of Arabidopsis lacks a functional violaxanthin deepoxidase, and therefore has no xanthophyll cycle. Lipid peroxidation can occur under low or moderate light when plants are subjected to an additional constraint on photosynthesis such as water deficit or chilling. Lipid peroxidation was more pronounced in the *npq1* mutant compared with the wild type, as measured by chlorophyll thermoluminescence, ethane production, and the total hydroperoxy fatty acids content when grown at warm temperatures (Havaux and Niyogi 1999). However, lipid peroxidation was amplified markedly under chilling stress, and photooxidative damage ultimately resulted in leaf bleaching and tissue necrosis in *npq1*.

Exposure of detached leaves or whole plants to a high photon flux density (1,000 μmol m^{-2} s^{-1}) and low temperature (10°C) results in PSII photoinhibition that is more severe in *npq1* than the wild type (Havaux and Kloppstech

2001). The loss of the xanthophyll cycle in *npq1* is associated with an increased susceptibility to photoinhibition under short-term photostress during chilling, but not during long-term photoacclimation. Thus, the increased photosensitivity of *npq1* at low temperature is linked to the inhibition of *n*on*p*hotochemical energy *q*uenching (NPQ) (Havaux and Kloppstech 2001). Chilling stress induced synthesis of Early Light-Inducible Proteins (ELIPs), which over time disappeared in *npq1* but remained stable in wild type plants (Havaux and Kloppstech 2001). Photoacclimation in *npq1*, resulted in a marked reduction of the light-harvesting pigment antenna of PSII and an increase of photosynthetic electron transport. In contrast, PSI appeared to be phototolerant to chilling stress. Over time at chilling temperatures PSII activity could recover in both *npq1* and wild type plants, suggesting that Arabidopsis is able to acclimate to chilling stress in the light without a functioning xanthophyll cycle (Havaux and Kloppstech 2001). It was also concluded that involvement of zeaxanthin in the protection of the chloroplast membranes against photooxidative damage does not appear to be the result of antioxidant activity related to quenching of chlorophyll in the light harvesting complexes (Havaux and Niyogi 1999). Taken together neither the xanthophyll cycle or the absence of zeaxanthin seem to have an essential protective function during long-term exposure of plants to excessive light energy, since the resulting inhibition of NPQ can be compensated by other adaptive mechanisms.

ELIPs are thylakoidal proteins that belong to the chlorophyll *a*/*b*-binding (CAB) protein family. An Arabidopsis mutant (*chaos*) affected in the posttranslational targeting of *l*ight-*h*arvesting *c*omplex (LHC)-type proteins to the thylakoids suppressed the rapid accumulation of ELIPs under high-light stress, resulting in leaf bleaching and extensive photooxidative damage (Hutin *et al*. 2003). The *chaos* mutant is defective in the function of the chloroplast signal recognition particle system that is involved in the rapid targeting of LHC-type proteins, including ELIPs to the thylakoid membranes. Wild type plants exposed to excess light energy induced by high photon flux density [1,000 µmol (photon) m^{-2} s^{-1}] and low temperature (7°C) exhibited a strong accumulation of both ELIP transcripts and proteins (Hutin *et al*. 2003). In contrast to wild type plants, *chaos* plants subjected to chilling stress at high light had extensive photooxidative damage leading to leaf bleaching. Constitutive expression of ELIP genes in the chaos mutant resulted in ELIP accumulation during light stress and restored the phototolerance of the mutant plants to that of the wild-type (Hutin *et al*. 2003). These findings link ELIPs with tolerance against chilling-induced photooxidation.

When exposed to photoinhibitory conditions, plants accumulate (ELIPs) which are considered to have a probable photoprotective function (Casazza *et al*. 2005). The Arabidopsis genome encodes two genes (*Elip1* and *Elip2*) that are differentially regulated. Normally, ELIPs accumulate during greening of etiolated seedlings. At 4°C Elip2 is strongly expressed. Using T-DNA insertion lines, the effects of ELIP mutants were assessed. It was found that phenotypically, single mutants for *elip1* or *elip2* were similar to wild type in their sensitivity to light- or light and cold-induced short-term photoinhibition in short-term experiments (Casazza *et al*. 2005). In contrast to other studies, it was concluded that in mature plants ELIPs do not have a direct role in photoprotection. Possibility treatments in the cold and the availability of a double *elip1*/*elip2* mutant will allow a more robust test of whether ELIPs are essential for Arabidopsis at low temperatures and high light.

Arabidopsis responds to excess light energy during chilling by accumulating carotenoid and flavonoid pigments and lipophilic antioxidants (Havaux and Kloppstech 2001). The simultaneous accumulation of these photoprotectants is likely to be an important component of the acclimation to chilling stress, which in turn reduces photooxidative stress in the chloroplasts. Analysis of flavonoid-deficient *tt* mutants revealed that UV/blue-light-absorbing flavonols possess a strong protective function against excess visible radiation. In contrast to the defect in *npq1*, the absence of flavonoids could not be overcome by a long term acclimation to chilling temperatures and high light by compensatory mechanisms (Havaux and Kloppstech 2001). Consequently, the inability to accumulate flavonoids results in extensive photooxidative and photoinhibitory damage to the chloroplasts (Havaux and Kloppstech 2001). The *tt5* mutant was shown to be the most light-sensitive of several flavonoid mutants. Following excess light for 6 days, PSII was more photoinhibited in *tt5* than *ttg* and *tt3* mutants. The wild type of ecotypes Columbia and Landsberg erecta show similar responses to chilling stress in high light. Increased lipid peroxidation was observed to occur in the *tt5* mutant. The mutants *ttg* and *tt3* were found to be less affected than *tt5* (Havaux and Kloppstech 2001). It is clear that the data from the *tt* mutants show that flavonoids protect plants from photoinhibition and photooxidation under excess visible light. The UV/blue light-absorbing flavonoids (absent in *tt5*) are more effective in preventing photoinhibition and photooxidation than the green light-absorbing anthocyanins (absent in all *tt* mutants) (Havaux and Kloppstech 2001). In the long-term, flavonoids appeared to be more effective photoprotectors than the xanthophyll-cycle carotenoids. The latter pigments seem to be relevant to the protection of plants against damaging effects of short-term photostress rather than long-term exposure to excess light energy (Havaux and Kloppstech 2001).

Transcriptional regulation at low temperatures

Arabidopsis plants cold acclimate and, in this process they show an increase in freezing tolerance in response to exposure to low non-freezing temperatures (Guy 1990; Thomashow 1999; Sung *et al*. 2003). Moreover, wild type Arabidopsis can remain at low temperatures for extended durations without apparent injury. The largest impact of growth at low nonfreezing temperatures is a reduction in the rate of growth and progression through the developmental stages of the life cycle. At the level of the gene, DNA microarray analysis studies have revealed that exposure of Arabidopsis plants to low temperatures of 4 or 13°C results in the up- or down-regulation of hundreds of cold-regulated (COR) genes (Fowler and Thomashow 2002; Kreps *et al*. 2002; Seki *et al*. 2002; Provart *et al*. 2003; Vogel *et al*. 2005). Many of the cold-inducible genes, including those involved in accumulation of osmolytes, cryoprotectants, antioxidant detoxification enzymes, chaperones, dehydrins, LEA proteins, enzymes involved in lipid, carbohydrate and secondary metabolism, transporters, and ABA and JA biosynthesis genes, are also commonly induced by exposure to other stresses, such as drought and high-salinity, which suggests the existence of cross-talk between low-temperature responses and other stress-signaling pathways (Zhu 2001; Kreps *et al*. 2002; Seki *et al*. 2002). In the light of these findings and of the occurrence of cross-tolerance mechanisms, it is very reasonable to hypothesize that at least some of the COR genes detected in Arabidopsis may also contribute in some manner to chilling fitness.

Cold acclimation in Arabidopsis involves the action of the C-repeat/dehydration responsive element Binding Factor (CBF) cold-responsive pathway (Thomashow 2001). This pathway includes cold-induced transient expression of the *CBF1*, *CBF2* and *CBF3* genes, which are transcriptional activators that bind to the C-repeat/dehydration-responsive element present in the promoters of downstream COR genes (Gilmour *et al*. 1998; van Buskirk and Thomashow 2006). Ectopic expression of *CBF1* or *CBF3* genes in transgenic Arabidopsis plants leads to constitutive expression of COR genes and enhanced freezing tolerance in the absence of cold acclimation (Jaglo-Ottosen *et al*. 1998; Gilmour *et al*. 2000). Although *CBF* genes play a key role in the acquisition of freezing tolerance, it is not yet clear whether the CBF family of transcriptional activators may also be in-

volved in regulation of genes important for chilling tolerance. Recently, Gong *et al*. (2002) identified an Arabidopsis mutant – *los4-1* (for low expression of osmotically responsive genes) – that exhibited impairment of cold-regulated expression of *CBF* genes and their downstream target genes, and that appeared to exhibit enhanced susceptibility to both freezing and chilling temperatures. The impaired chilling resistance was manifested by increased electrolyte leakage rates and leaf death symptoms after 2 weeks of growth at 4°C in the dark or after prolonged growth for up to 2 months at 4°C in the light. Overexpression of *CBF3* under a strong constitutive promoter was able to restore chilling resistance in the *los4-1* mutants (Gong *et al*. 2002). Overexpression of *CBF1* also appears to increase chilling tolerance in chilling-sensitive tomato plants (Hsieh *et al*. 2002; Lee *et al*. 2003). In any case, the possible roles, if any, of the CBF transcriptional activators in governing chilling-tolerance responses await further detailed investigation.

Although the CBF cold response pathway is currently the best understood genetic system involved in cold acclimation, it is not the only such pathway; there are additional CBF-independent cold response pathways. In fact, Fowler and Thomashow (2002) reported that only about 12% of the detected cold-responsive genes could be assigned to the CBF regulon, and they identified 15 other cold-regulated transcription factors that appear not to participate in the CBF cold-response pathway. Seki *et al*. (2002) also identified 40 transcription factors that regulate stress-inducible genes that belong to AP2/EREB, zinc finger, ERF, WRKY, bZIP and MYB families. In this context, Kim *et al*. (2001) isolated a soybean cDNA encoding a C_2H_2-type zinc finger protein – *SCOF-1* – that is specifically induced by low temperature and abscisic acid, but not by other stresses. Constitutive overexpression of *SCOF-1* in Arabidopsis plants induced COR gene expression and enhanced cold tolerance. Another gene that might be involved in governing transcriptional regulation at low temperatures is *Osmyb4*, a myb-type transcription factor that in rice is specifically induced by low temperatures (Vannini *et al*. 2004). Overexpression of *Osmyb4* in Arabidopsis significantly increased cold and chilling tolerance, and activated COR gene expression (Vannini *et al*. 2004).

Overall, the complex network of transcriptional regulation at low temperatures and the possible interrelationships between cold-regulated gene expression and acquisition of chilling tolerance is just beginning to be elucidated. In the future, identification of more mutants and T-DNA knockouts with chilling susceptibility phenotypes, and further DNA microarray studies will certainly increase our basic understanding regarding the molecular control of cold-regulated gene expression and its roles in the acquisition of low-temperature tolerance.

Programmed cell death and chilling stress

Programmed cell death (PCD) is a common process in eukaryotes with functions in development and in response to pathogens and other forms of stress. PCD is often triggered by a number of different abiotic stresses, including high or low temperature, water stress and UV irradiation (Beers and McDowell 2001). The Bax inihibitor-1 (BI-1) is thought to be a cell death suppressor that is conserved in both animals and plants. Two T-DNA insertions in the Arabidopsis *AtBI1* gene were examined and it was found not to be essential for normal vegetative growth and development in Arabidopsis suggesting a specialized function in plants in response to stress (Watanabe and Lam 2006). Therefore the phenotype of the two mutant lines, *atbi1-1* and *atbi1-2*, with a C-terminal mis-sense mutation and a gene knockout, respectively, was not different from wild-type plants under normal growth conditions. Upon infiltration of leaf tissues with the fungal toxin fumonisin B1, PCD was amplified. In addition, these two mutant lines exhibited accelerated progression of cell death and increased sensitivity to heat shock-induced cell death (Watanabe and Lam 2006). In wild-type plants under these two conditions, expression of *AtBI1* mRNA was up-regulated prior to the activation of cell death, suggesting that the increase of *AtBI*1 expression is important for basal suppression of cell death progression (Watanabe and Lam 2006). Over-expression of *AtBI1* in the two mutant backgrounds under the two elicitation conditions restored a reduced induction of programmed cell death phenotypes. However, over-expression of *AtBI1* did not significantly delay cell death induced by either the fungal toxin or heat shock treatments (Watanabe and Lam 2006). It thus seems likely that an increase in *AtBI1* expression is required for basal suppression of cell death progression during stress in wild-type Arabidopsis. These results indicate a direct genetic link for a role of BI-1 as an attenuator for cell death progression triggered by both biotic and abiotic types of cell death signals in Arabidopsis.

Chilling injury frequently is manifested by the formation of lesions, of regions of necrosis and cell death. BI-1 is thus arguably the best candidate for a conserved 'core regulator' of PCD in plants. It would be very interesting to determine whether loss of function lines for BI-1 in Arabidopsis would have elevated sensitivity to chilling conditions and show increased levels of necrotic lesions.

When nontransgenic tomato plants were transferred to 4°C with normal lighting conditions, necrotic lesions appeared in 3 or 4 days and necrosis became more severe over time. To explore the role PCD in chilling stress, transgenic tomato plants over-expressing animal antiapoptotic genes *bcl-xL* and *ced-9* were tested for chilling tolerance (Xu *et al*. 2004). Over-expression of *bcl-xL* and *ced-9* specifically reduced the formation of necrotic lesions, but not other symptoms of chilling stress in tomato leaves during exposure to 4°C (Xu *et al*. 2004). Thus, plants expressing *bcl-xL* or *ced-9* showed an improved tolerance to chilling stress at 4°C. High-expressing plants, although stunted, were most effectively protected. In addition, high expression of *bcl-xL* or *ced-9* affected plant growth and seed development in the absence of stress. An additional positive effect of over-expression of *bcl-xL* and *ced-9*, was a dramatically delayed temperature-induced leaf senescence at 7°C, where it was concluded that high levels of anthocyanins accumulated may have helped to limit oxidative stress (Xu *et al*. 2004).

CONCLUSIONS

Low temperature is a major environmental factor that causes massive losses of commercial horticultural crops; limits the geographical distribution of tropical and subtropical commodities, and imposes major limitations on the postharvest handling of fruits and vegetables. In contrast to chilling-sensitive species, the cruciferous plant *Arabidopsis thaliana* is chilling tolerant, and is able to grow to maturity even at very low chilling temperatures. Thus, at the genetic level, Arabidopsis may provide a useful genetic source for use in the identification of chilling tolerance traits, and may serve as an appropriate model system for elucidation of the molecular mechanisms involved in imparting chilling resistance.

In the present review, we have summarized some of the principle advantages of using Arabidopsis as a model genetic system in studying chilling tolerance mechanisms in plants and provided a few examples. First, the availability of EMS mutagenized and T-DNA knockout chilling-sensitive mutants will, in the future, enable the isolation of additional genes essential for growth and survival at low temperatures (Schneider *et al*. 1995a; Tokuhisa *et al*. 1997). The various *chs* mutants that have been identified have a wild-type appearance at normal temperatures, but show damage when transferred to chilling temperatures (**Fig. 1**). Therefore, any biochemical difference detected in comparisons between the chilling-tolerant and the *chs* mutants must reflect the presence of a basic component related to chilling tolerance. One possibility is that the *chs*-mutated genes encode specific proteins crucial for survival under chilling conditions but

are not required at higher temperatures (Hugly *et al*. 1990). On the other hand, it is also possible that some of the *chs* genes are mis-sense mutations that encode polypeptide products that function adequately at normal (permissive) temperatures but lose their function at low (non-permissive) temperatures (temperature sensitive mutants). Such a mis-sense mutation in an essential gene might lead to a chilling-induced phenotype (Tokuhisa *et al*. 1997). In any case, cloning of the *chs* mutant genes is expected to improve our basic understanding regarding the molecular mechanisms and biochemical pathways required for chilling resistance.

Second, Arabidopsis has proven to be a useful model genetic system for the identification of genes involved in fatty acid biosynthesis and desaturation (Somerville and Browse 1991). Identification of mutants deficient in their fatty acid composition, and cloning their genes enabled exploration of the biochemical pathways involved in fatty acid metabolism, and proof of their roles and importance in low-temperature fitness.

Third, using Arabidopsis as a model system has allowed researchers to explore major biochemical pathways and mechanisms involved in protection of the photosynthesis system from photooxidative damage that might occur following exposure to high photon flux density under low temperatures. Mutant analysis and functional genetic studies have revealed the importance of flavonoids, xanthophyll-cycle carotenoids and ELIPs in protecting plants against photo-stress and exposure to excess light energy.

Fourth, microarray gene expression studies in Arabidopsis enable researchers to explore the complex transcriptional regulation network involved in cold-induced gene expression, and to evaluate its possible contribution to freezing and/or chilling tolerance.

In the future, further cloning and isolation of *chs* mutant genes, together with genome-wide transcriptional analysis studies of wild-type and *chs*-mutant plants under optimal and chilling temperatures should lead to further improvement of our basic understanding regarding the molecular mechanisms involved in the acquisition of chilling resistance. Parallel functional-analysis studies by over-expression or down-regulation of specific putative chilling-tolerance genes and evaluation of their effects on chilling resistance or susceptibility will enable us to directly prove their roles in and their contributions to low-temperature fitness.

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