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Plant Cold Acclimation: The Role of Abscisic Acid

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Abstract

The freezing tolerance or cold acclimation of plants is enhanced over a period of time by temperatures below 10°C and by a short photoperiod in certain species of trees and grasses. During this process, freezing tolerance increases 2–8°C in spring annuals, 10–30°C in winter annuals, and 20–200°C in tree species. Gene upregulation and downregulation have been demonstrated to be involved in response to environmental cues such as low temperature. Evidence suggests ABA can substitute for the low temperature stimulus, provided there is also an adequate supply of sugars. Evidence also suggests there may be ABA-dependent and ABA-independent pathways involved in the acclimation process. This review summarizes the role of ABA in cold acclimation from both a historical and recent perspective. It is concluded that it is highly unlikely that ABA regulates all the genes associated with cold acclimation; however, it definitely regulates many of the genes associated with an increase in freezing tolerance.

Key words: Freezing tolerance; Gene induction; ABA responsive element; Hormones

Introduction

Cold acclimation, a process in plants that requires days to weeks for full development, is induced primarily by temperatures below 10°C, and by a short photoperiod in certain species of trees and grasses. Acclimation proceeds in either 1, 2, or 3 stages, depending on the species. The first phase involves primarily acclimation to low temperature (LT) growth and upregulation of transcriptional factors. During this stage of acclimation, plants achieve tolerance to short frosts of −5°C to −9°C. Annual plants like petunia, canola, and oat show this type of acclimation. The second stage is in response to temperatures that approach 0°C. During this stage cryoprotective compounds (such as proteins and sugars) are produced, and repair mechanisms develop. Winter annuals and perennials such as winter cereals, grasses, and trees fall into this category, and some possess the potential to tolerate temperatures as low as −196°C for extended periods. These very hardy species survive sub-zero temperatures by tolerating freeze-induced desiccation. A third phase of acclimation can occur at temperatures below 0°C in some plants, resulting in

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a further increase in the freezing tolerance. This phase is a combination of both biochemical and biophysical events.

In spite of a vast amount of research, it is still not clear how plants perceive the environmental signal for the induction of freezing tolerance for the above phases. It is also unknown if each stage of acclimation requires a separate initiation cue, or if subsequent stages are initiated by the previous stage. Master switch genes are postulated to regulate a myriad of genes associated with increases in freezing tolerance (Thomashow 1999). However, what regulates these master switches has only been hypothesized to date. Some evidence suggests that the phytohormone abscisic acid (ABA) can substitute for the LT stimulus (Chen and Gusta 1983), whereas other studies have demonstrated either little or no effect of ABA on the development of freezing tolerance. It is also unknown whether ABA may be involved in all three phases of cold acclimation or primarily in the first stage. Because of conflicting results, several authors suggest there may be ABA-dependent and ABA-independent pathways associated with cold acclimation. Classical and molecular genetics, including reverse genetics in combination with biochemistry and biophysics, can reveal how ABA is involved in cold acclimation in contrast to correlative studies on gene expression. A great deal of research in the area of LT tolerance has been conducted on the model plant *Arabidopsis thaliana*, which has a limited cold hardiness potential. *Arabidopsis* is probably not a good model in which to study cold acclimation of hardy species that have a second stage of acclimation. The process is further complicated because many factors such as wounding, mechanical shock, water stress, light, and cold shock can induce 3–5°C of freezing tolerance, which may not involve the same genes that are involved in cold acclimation.

This review summarizes the role of ABA in cold acclimation from both a historical and a recent perspective. It describes some of the pitfalls associated with the application of exogenous ABA, and it suggests why the results obtained from ABA-dependent studies can be variable.

The Early Work

It was probably research on the environmental cues that induce cold acclimation in woody species that led to the suggestion that ABA was involved and thus initiated subsequent studies on the role of ABA in abiotic stress. Irving and Lanphear (1967) and Van Huystee and others (1967) proposed a two-step acclimation process for woody species. Irving and Lanphear (1967) verified that the induction of freezing tolerance was truly a photoperiodic phenomenon, by exposing English ivy plants to short days including 1-h night interruption during the 16-h dark period. The night interruption treatment resulted in a level of freezing tolerance in short days similar to that experienced by plants exposed to long days. These experiments laid the groundwork for the latter suggestion that phytochrome was involved in induction of the cold acclimation process. This was later verified for other woody species (McLeester and others 1969; Howell and Weiser 1970; Fuchigami and others 1971b). Short photoperiods combined with warm temperatures trigger the first stage of acclimation (Fuchigami and others 1971a,b; Howell and others 1970; Pakitina 1968), in which a flush of metabolic activity occurs (Levitt 1972; Weiser 1970). The second phase is induced both by exposure to low non-freezing temperatures, and later it was discovered that the third phase is induced by exposure to sub-zero temperatures (Weiser 1970).

Chandler (1954) postulated that substances produced during the initial stages of acclimation were translocated from leaves into the bark to induce cold acclimation. Irving and Lanphear (1967) were the first to demonstrate that under long days and natural fall temperatures a cold hardiness inhibitor was produced in leaves of woody species, as removal of leaves exposed to long days accelerated cold acclimation. However, the inhibition by long days could be overcome by cool acclimating temperatures. Most interesting were split-plant studies by Irving and Lanphear (1967), which demonstrated that a cold hardiness–inducing substance was produced in the leaves of the short-day exposed branch that was subsequently translocated to the long-day exposed branch, enhancing freezing tolerance at non-acclimating temperatures. From this experiment the authors concluded that the translocated substance, produced under short days, was a hormone, although Steponkus and Lanphear (1967) suggested the substance might also be a sugar. The role of sugars in gene regulation will be discussed later. Subsequently, Irving and Lanphear (1968) reported plants of *Acer negundo*, sprayed with gibberellic acid (GA) under short days, did not acclimate, whereas plants sprayed with GA inhibitors showed a significant increase in cold hardiness. At that time a recently identified hormone, dormin, was shown to be a GA antagonist (Chrispeels and Varner 1967; Thomas and others 1965). The application of dormin to plants exposed to long days resulted in an increase in freezing tolerance equivalent to those

exposed to short days (Irving and Lanphear 1968). In lettuce seed germination assays, high levels of an inhibitor similar to dormin were produced in leaves exposed to short days. Dormin was later renamed abscisic acid (ABA). To the authors' knowledge, the work by Irving and Lanphear was the first to demonstrate that ABA was involved in cold acclimation; however, it was Wright and Hiron (1969) who first suggested that ABA was a stress hormone.

Exogenous ABA—Limited or No Acclimation of Whole Plants

Irving and Lanphear (1968) induced stems of *Acer negundo* to harden from –12°C to –21°C when leaves were fed 100 mg^{-1} of racemic ABA under long days. Fuchigami and others (1971a), working with explants of Red Osier dogwood, could not detect any discernible difference in freezing tolerance between ABA-treated plants and the controls. Limited results were also obtained by foliar application of ABA to citrus seedlings (Young 1971), *Chrysanthemum morifolium* rhizomes (Fayyaz and others 1978), winter wheat (Gusta and others 1982), apple seedlings, and peach and apricot trees (Holubowicz and others 1982). Moderate increases in freezing tolerance were obtained when hardened winter wheat plants were sprayed with ABA (Lalk and Dörfling 1985) and ABA analogs (Flores and others 1988) 24–48 h prior to freeze tests. In contrast to foliar applications, ABA or ABA analogs supplied aseptically to stem-cultured potato plants (Chen and others 1979), embryogenic cell lines of *Picea abies* (Sabala and others 1996), and alfalfa grown hydroponically (Rikin and others 1975), and as a root drench to rye seedlings (Churchill and others 1988), resulted in a significant increase in freezing tolerance. In contrast to a foliar spray, root application at non-acclimating temperatures increased the freezing tolerance of rye seedlings from −3°C to −9°C after 3 days of exposure. By day 6, the effects of the ABA application diminished, and freezing tolerance decreased to −6°C. None of these studies demonstrated that ABA application resulted in the same level of freezing tolerance as low temperature induction.

The origin of differences in cold acclimation between foliar application of ABA versus a root drench or uptake by stem cuttings must relate to uptake, partitioning, or vascular transport, or combinations thereof, because the site of action (that is, the putative receptor) is likely to be the same (Churchill and others 1998). The leaf's cuticle is the major barrier to foliar ABA uptake (Blumenfeld and Bukovac 1972); also, ABA is readily inactivated by light. It appears that root drench and stem cutting applications cause ABA to be rapidly and efficiently translocated to the active site. As ABA enters into the cell, the enzymes for its catabolism are induced and (+)ABA is oxidized to (−) phaseic acid (PA) or conjugated irreversibly with glucose to form a glucose ester (Oritani and Kiyota 2003). In wilted bean leaves the half-time of turnover of ABA to PA was about 3 h (Harrison and Walton 1975). Neither PA nor the ABA glucose ester was effective in inducing freezing tolerance in plant cells (Churchill and others 1994; Robertson and others 1994). This may explain the transient nature of the increase in freezing tolerance in plants that is induced by exogenous ABA.

Exogenous ABA—Dramatic Increases in Freezing Tolerance: Cell Cultures

The studies that have been described demonstrated that exogenous ABA induced a limited amount of freezing tolerance for a short time; however, the extent of induced freezing tolerance was invariably less than that which resulted from exposure to low temperatures. For example, Churchill and others (1994) increased the freezing tolerance of Puma rye to −9°C with a root drench of 100 μM ABA; however, Puma rye tolerates −30°C when fully acclimated in response to low temperatures. Results of this nature led other scientists to suggest that either ABA is not involved in the induction of freezing tolerance or that there was a second independent pathway. Chen and Gusta (1983), working with cell suspension cultures of bromegrass, winter wheat, rye, carrot, and triticale, found that racemic ABA replaced the low temperature requirement for cold hardening, but not in species incapable of hardening in response to low temperatures. Cells of rye and bromegrass, which are capable of hardening, tolerated −32°C to −34°C after 4 days of incubation in 100 μM (±)–ABA at 20°C. This level of hardiness is comparable to that which results from natural hardening. Furthermore, if cells were subcultured every 7 days in 100 μM (±)–ABA, there was no loss in freezing tolerance (Robertson and others 1995). However, if cells were transferred to a medium without ABA, they dehardened to control levels. Recently, Ishikawa (personal communication) demonstrated that cell cultures of rice treated with ABA tolerated temperatures as low as −30°C.

Reaney and Gusta (1987) observed that, if ABA was dissolved in dilute KOH instead of ethanol, and if the ratio of the cell inoculum to the ABA con-

centration was optimum, bromegrass cell cultures tolerated liquid N_2 temperatures (−196°C) after 7days in 100 μM (±)−ABA. Reaney and others (1989) also confirmed Irving and Lanphear's (1968) earlier report that GA inhibited ABA induction of freezing tolerance. GA_4, GA_7, and GA_9 were equally effective at inhibiting freezing tolerance induced by ABA; GA_3 had little effect. The ratio, rather than the absolute amounts of ABA and GA appeared to be critical for the development of freezing tolerance. Wilen and others (1994) noted a synergism between ABA and jasmonic acid in inducing freezing tolerance in bromegrass cell cultures. Abscisic acid is also known to interact with sugars and with nitrogen (Kang and others 2004). Abscisic acid/sugar interactions will be discussed later. Racemic ABA, which is a 50:50 mixture of the (+) or S-ABA (natural form) and the (−) or R-ABA (unnatural or analog form), is most often used as an exogenous source of ABA. As will be discussed, the unnatural form is not as active as the natural form, but it does upregulate many of the same genes as (+)-ABA. (−)-ABA has a stronger inductive effect than (+)-ABA on a subset of weakly ABA-inducible genes (Cutler, personal communication). Also, (−)-ABA is catabolized much more slowly than (+)-ABA, and it may also induce the biosynthesis of (+)-ABA (Abrams, personal communication). The cost of (+)-ABA is no longer prohibitive for its routine use in assays. Furthermore, continued use of the racemic mixture of ABA in experiments only leads to confusing results.

If ABA is involved in natural cold acclimation, why is there only a transient increase in hardiness of foliage of potato (Chen and others 1983), spinach (Guy and Haskell 1988), and *Arabidopsis* (Lang and others 1994)? To establish the involvement of ABA in cold acclimation, it is necessary to measure the metabolism of ABA and thereby determine the relative rates of ABA synthesis and catabolism. For example, ABA biosynthesis is reported for the maintenance of thermodormancy of lettuce seeds (Yoshioka and others 1998). Chiwocha and others (2003) detected a transient increase in ABA in thermodormant seeds, but thereafter the levels remained constant for 8 days. However, analysis of the ABA metabolites revealed continuous synthesis and turnover of ABA. These findings were consistent with the report by Yoshioka and others (1998) that ABA levels did not change in thermodormant lettuce seed, but that ABA synthesis continued to occur at a rate sufficient to maintain dormancy. Although it is possible to quantitate the total amount of ABA in tissue, it is still not known what the critical concentration is at the active site or what the active site is.

The question remains, why does ABA not totally substitute for low temperature in hardy species? It has long been established that sugars, predominantly sucrose, are intimately involved in freezing tolerance (Levitt 1972). Wanner and Junttila (1999) demonstrated that a combination of cold and light is required to induce freezing tolerance in *Arabidopsis* leaves, and this combination is associated with the accumulation of soluble sugars. At low temperatures in the absence of light, all of the COR (cold-responsive) genes were upregulated; however, freezing tolerance was not enhanced. The authors concluded that sugar accumulation is a fundamental component of enhanced freezing tolerance and acts in concert with the COR gene products. Robertson and others (1994a) also reported that stress-tolerance mechanisms in ABA-treated cells develop as a result of cooperative interactions between ABA-induced stress proteins and sucrose. A brief washing of bromegrass cell cultures incubated with 75 μM ABA for 4 days resulted in a 15–20°C loss in freezing tolerance (Ishikawa and others 1990). However, addition of 10% sucrose, 30 min prior to freezing restored the freezing tolerance of the cells. The apoplastic fluid of plants contains negligible levels of solutes—for example, sucrose; however, when plants are frozen slowly, solutes from the symplast accumulate in the apoplast and act as cryoprotectants (Olien 1984). The principal solutes are sucrose and fructose, which interact with extracellular ice either to prevent adhesions to the plasma membrane or to moderate the rate of ice growth (Reaney and Gusta 1999). Infrared videothermography revealed that the freezing rate of apoplastic extracts from canola leaves was primarily dependent on simple sugars and not on proteins (Gusta and others 2004). Livingston and Henderson (1998) also reported that apoplastic sugars and fructans increased in the second phase of cold acclimation. Addition of sucrose to non-ABA-treated cells has no effect on freezing tolerance (Ishikawa and others 1990). Robertson and others (1994a) suggested that an ABA-responsive protein in the plasma membrane binds sugars and that this combination confers stability or protects the plasma membrane from freeze-induced dehydration.

Further evidence for the involvement of sugars comes from a study in which (+)-ABA, the natural form and (−)-ABA, the unnatural form, induce dehydrin gene expression and the accumulation of COR proteins, but (−)-ABA-treated cells only tolerated −19°C, whereas the (+)-ABA-treated cells survived to below −40°C (Robertson and others 1994a; Wilen and others 1996). The major difference exhibited by the two forms of ABA was that

(+)-ABA stimulated sucrose uptake from the medium, whereas (−)-ABA did not. How does all of this relate to whole plants? When plants are treated with exogenous phytohormones not all of the events associated with the natural reaction are induced. Actively growing plants would not have high levels of sugars compared to plants grown at low temperatures. The application of sugars with ABA does not guarantee uptake or translocation to the active site as would occur naturally. Therefore, although COR proteins are produced in response to exogenous application of ABA, the lack of sugars present in the plant may limit the development of freezing tolerance.

ABA and Phytochrome

As mentioned above, the short day perception of cold acclimation of trees was demonstrated over 35 years ago by several research groups (see review by Weiser 1970). Recent studies have demonstrated that overexpression of the oat (*Avena sativa*) phyA gene in hybrid aspen (*Populus tremula* × *P. tremuloides*) changes the critical day length and prevents cold acclimation under short days (Olsen and others 1997). In contrast, reduction of PHYA results in increased sensitivity to photoperiod (Eriksson 2000). Exposure of the lines overexpressing PHYA to 4°C resulted in cold acclimation comparable to the wild type (Welling and others 2002). Similar levels of ABA were measured in the line overexpressing PHYA under both long days and short days when grown at 18°C, although ABA was twofold lower than in the wild type. Exposure to 0.5°C resulted in a dramatic increase in ABA in both the wild type and the line overexpressing PHYA. The authors concluded that phytochrome A is involved in the photoperiodic regulation of ABA, but low temperature regulates ABA by a different mechanism. Both short day and low temperature are perceived by leaves of silver birch (*Betula pendula*), which results in enhanced freezing tolerance. A dehydrin was isolated from the leaves exposed to low-temperature dehydration, salt, and exogenous ABA (Puhakainen and others 2004). Short day exposure resulted in a minimal increase in the transcripts for this dehydrin; however, if the short day treatment was followed by low temperatures, the transcripts significantly increased. These results suggest that the short photoperiod potentiates the low temperature response. This supports the earlier suggestion by Weiser (1970) that translocatable hardiness promoters produced in leaves exposed to short days sensitize cells to perceive the low temperature cue. In birch, both short days and low temperature increase endogenous ABA (Li and others 2002) that could operate in concert with low temperature to upregulate dehydrins. Dehydrins have been closely associated with increase in freezing tolerance of trees (Arora and Wisniewski 1994; Wisniewski and others 1996) and herbaceous plants (Robertson and others 1994b). Balk and others (2004) found that of the top 50 genes that had the highest correlation with a functional shift in cold hardiness, 18 were dehydrin genes.

ABA and Sugars

Rock (2000) postulated that tissue-specific gene expression regulated by ABA and other developmental and environmental pathways relies on the combinatorial action of a large number and variety of transcription factors. Genetic screens for sugar sensing/response and osmotic response mutants revealed links between response pathways for ABA, soluble sugars, and osmotic stress. For example, low concentrations of exogenous sugars overcome germination inhibition by ABA (Finkelstein and Lynch 2000). In contrast, high concentrations of sugars (> 300 mM) can inhibit seedling growth in an ABA-dependent manner, and not simply as a result of the osmotic effects of sugar (Gibson 2004). Numerous results have been obtained with transcriptional mutants that provide evidence that ABA and sugar can act either in parallel or in intersecting pathways (Finkelstein and others 2002). For example, high glucose induces both ABA synthesis and expression of ABA transcriptional factors, which led to the suggestion by Arroyo and others (2003) that altered ABA levels or ABA responses may mediate some aspects of sugar signaling. Considering the long known cryoprotective involvement of sugars in cold acclimation, it is tempting to speculate, with this new evidence, that the role of sugars may be more than cryoprotective, as suggested 38 years ago by Steponkus and Lanphear (1967).

Sugar signal transduction is tightly linked with ABA and processes affected by ABA, such as osmotic stress and ethylene signaling (Cheng and others 2002). Glucose and ethylene signaling pathways antagonize each other, and that antagonism may be mediated in part by ABA (Zhou and others 1998). Glucose has been shown to have direct and extensive control of ABA biosynthesis (León and Sheen 2003), whereas high concentrations of glucose result in high endogenous levels of ABA (Arenas-Huerters and others 2000), because of increased transcripts of ABA biosynthesis genes (Cheng and

others 2002). Sucrose is also postulated to regulate ABA biosynthesis (Leon and Sheen 2003). AB14, which encodes a transcription factor of the APETALA 2 (AP2) domain family, is activated by sugars in an ABA-dependent manner (Cheng and others 2002). This demonstrates the involvement of sugars in ABA signaling genes. The AB15 gene, which confers hypersensitivity to sugars (Brocard and others 2002), encodes a transcription factor that is a member of a large basic leucine zipper (bZIP) domain family that is highly inducible by ABA and mannitol. Members of the AB15 family have been shown to be ABA and/or stress inducible. Recently, Avonce and others (2004) showed that a low level of accumulation of trehalose (glucose-glucose) in *Arabidopsis* plants overexpressing trehalose synthase resulted in enhanced desiccation tolerance. Although the level of accumulation was deemed insufficient for the action as an osmoprotectant, the transgenic plants were more desiccation tolerant than the wild types. Trehalose has long been implicated in abiotic stress such as drought, salt, heat, and freezing tolerance. In transgenic plants overexpressing trehalose synthase, the ABA transcriptional factor AB14 is upregulated; however, in the presence of glucose it is downregulated.

ABA Regulation

Abscisic acid activates a wide array of genes associated with low temperatures, drought, salinity, heat wounding, desiccation, storage proteins, dormancy, germination, the arrest of embryonic development, and the closure of stomates (Giraudat and others 1994; Busk and Pages 1998). Abscisic acid-responsive genes contain in their promoter one or several copies of the DRE/CRT cis-element (drought responsive element/c-repeat transcription element), which contains an ABA-responsive element (ABRE) that has the core sequence CCGAC (Bark and Pages 1998). For the induction of cold acclimation, a family of cold-inducible transcription factors referred to as ''C-repeat binding factors (CBFs),'' bind to this element, initiating the transcription of downstream cold-responsive genes. Initially, three CBFs were found to be involved in cold acclimation, CBF1, CBF2, and CBF3, which are equivalent to the dehydration-responsive elements DREB1B, DREB1C, and DREB1A, respectively (Liu and others 1998). These are members of the apetala 2/ethylene-responsive element-binding protein (AP2/EREBP) family of transcription proteins. To add to the confusion, there is also a DREB2 family that is involved only in drought/osmotic stress (not cold) through the CRT/DRE element (Liu and others 1998). It is not known what upstream factor regulates the CBFs; however, there is speculation that its induction could be due to a biophysical response. Several studies have shown a transient increase in cellular Ca^{2+} prior to the activation of ABA and cold-inducible genes (Knight and others 1996, Thomashow 1999); however, it is not known what the specific molecular components are that mediate Ca^{2+}-associated gene activation. The major Ca^{2+} sensors in plants include calmodulin (CAM) (Zieliniski 1998), CAM domain-containing protein kinases (CDPKs) (Herman and others 2000), and calcineurin B-like calcium sensors (CBLs) (Luan and others 2002). CDPKs act as both Ca^{2+} sensors and kinases, whereas CAMs and CBLs are small Ca^{2+}-binding proteins that do not have enzymatic activity and that function by interacting with their target proteins. A constitutively active form of CDPK activates ABA and a stress-responsive gene promoter in maize leaf cells (Sheen 1996), as well as overexpression of a CDPK upregulated stress-related gene in rice (Saijo and others 2000). CBLs target a family of protein kinases referred to as CIPKs (CBL-interacting protein kinases) (Luan and others 2002). The expression of CIPK3 is responsive to exogenous ABA and cold, salinity, and wounding, but not to drought (Kim and others 2003). This has led to the suggestion that CIPK3 is involved in cross adaptation because it may mediate the interaction of ABA in the abiotic stress signal-transduction pathways.

Novillo and others (2004) recently reported that CBF2 negatively regulates CBF1/DREB1B and CBF3/DREB1A. With a reverse genetic approach, the authors identified a mutant, cbf2, in which the CBF2/DREB1C gene was disrupted. The cbf2 mutant had increased expression of CBF1/DREB1B and CBF3/DREB1A, as well as increased freezing, dehydration, and salt tolerance compared to the wild type.

Controlled Environment Acclimation versus Natural Acclimation

Understanding the relationship between cold acclimation initiated in a controlled environment chamber and the process of acclimation that occurs under field conditions can be problematic. The expression of PpDhn1, a peach dehydrin gene, can serve as a case in point. This dehydrin gene has previously been shown to be responsive to low temperature, drought, and ABA (Artlip and Wisniewski 1997; Artlip and others 1997). In studies in a controlled environment chamber, the

dehydrin gene was induced in response to low temperature, whereas 3 weeks of short days at 20°C had no impact on the gene (Wisniewski unpublished results). This pattern of regulation is hard to reconcile with seasonal expression patterns in the field, where expression of the dehydrin gene is first observed in August and increases during the fall. Although short photoperiods are present at this time of year, low temperatures (5°C) are not. Perhaps the expression of the dehydrin gene is more affected by the cycling of warm and cool temperatures than by a specific low temperature. Another possible factor is that the light spectrum in a controlled environment chamber would not be the same as in sunlight. There is good evidence that plants use photoreceptors to receive light signals and transduce them to modulate light-responsive gene expression (Jiao and others 2003). Far-red and red light via the phytochrome system (Green and Tobin 2002) and blue light, mainly through the cytochromes (Jiao and others 2003) and phototropins, regulate the genome through a transcriptional cascade. Over 20% of the transcription factor genes in *Arabidopsis* are responsive to blue-light exposure, with 219 and 115 genes upregulated or downregulated, respectively (Jiao and others 2003). At high latitudes, the long periods of twilight rich in blue and far-red energy influence plant development differently than at lower latitudes (Shropshire 1972). Recently, Usami and others (2004) found a strong synergistic interaction between phytochromes and cryptochromes for the blue-light response. In northern latitudes, the decline in the red portion in the spectrum, relative to the far-red, during sunrise and sundown, may be accentuated by the longer twilight period and the lower angle of incidence of the sun (Robertson 1966). McKenzie and others (1974) demonstrated a marked promotion of cold acclimation in Red Osier dogwood by an end-of-day far-red light treatment, which reduces the critical night length for the short day induction. At any rate, this is just one example that indicates how we must be very careful when trying to extrapolate our research results beyond the conditions that generated the data.

It is suspected that in many studies what the researchers are measuring is an injury response and not an application response. A similar situation exists for plants transferred from 20°C to 4°C to study genes initially involved in frost acclimation. The roots of plants in pots cool at a much quicker rate than experienced in nature, where there is a massive volume of soil. Plants transferred from 20°C to 4°C wilt in response to a decrease in the hydraulic conductivity of the roots. This results in a transient increase in ABA, followed by a decrease to near the basal level. Preliminary proteomic analysis has revealed that more than 40 new proteins were increased in plants exposed to a cold shock, but not in plants cooled slowly to 4°C over a 48-h period (Trischuk and Gusta, unpublished results). In addition to low temperature, wounding, mechanical shock, drought, and osmotic stress can also induce members of the CBF family. In response to a cold shock, CBFs transiently increase for 24–48 h and thereafter return to basal levels (Thomashow 1999). However, when plants are exposed to natural cold-acclimating conditions, CBF is highly upregulated for 4–6 weeks (Trischuk personal communication). Another example is the addition of 20–200 mM salts to the soil or hydroponic solution, which causes a dramatic osmotic shock. In nature, salts accumulate slowly in the soil surface layer, which allows the plant to perceive the signal for salinity acclimation prior to exposure to high concentrations. Drought studies involving withholding water to plants in pots or excising branches that are left to dry on the laboratory benches do not represent drought acclimation. There is insufficient time for the plants to acclimate to such rapid water loss. In nature, plants are initially subjected to a mild water stress during the day, which is followed by a recovery period during the night. This cycle continues each day, and the intensity increases unless it is relieved by rain or irrigation. It is during this cycling that drought-responsive genes are upregulated. In pots for acclimation, the loss of water is constant because of the non-cyclic nature of growth conditions. In addition, soil or the growth medium in a pot does not possess the typical soil structure present in natural conditions, and water in pots is lost at a much more rapid rate than in field conditions.

Similarly, the addition of 100 μM ABA applied as a foliar spray or to the roots is also a shock. This is somewhat analogous to a person taking medication for a headache. A person is quite comfortable with 1 or 2 two aspirins but may suffer extensive neuron damage from 100 aspirins. Such ''shock and awe'' experiments may not truly reflect what genes are involved in stress acclimation.

ABA-Dependent and ABA-Independent Pathways

Because there is considerable overlap in the expression pattern of stress genes associated with cold,

drought, salinity, and ABA, it was initially suggested that environmental cues and ABA share common elements in the signaling pathways (Thomashow 1999; Shinozaki and Yamaguchi-Shinozaki 2000). Further research suggested that there was one ABA-dependent and one ABA-independent cold-acclimation pathway (Thomashow 1999; Shinozaki and Yamaguchi-Shinozaki 2000). Conclusions were based on the observation that the transient increase in ABA in response to cold was not sufficient to induce ABRE-containing genes. Another example was cold-inducible (COR/RD/KIN) genes, which contain the DRE/CRT cis-element that is responsible for cold-induced gene expression but not for ABA induction, whereas ABA-induced expression requires a different cis-acting element called ABRE (Thomashow 1999; Shinozaki and Yamaguchi-Shinozaki 2000). This led Thomashow (2001) to conclude ABA had little or no role in the induction of cold tolerance, in spite of previous results to the contrary. In many studies involving exogenous ABA, the concentration of ABA in the plant was not determined, nor was its rate of catabolism or the expression of known ABA-responsive genes. The transcripts for the CBF transcriptional activator also known as DREB1) proteins have recently been shown to increase in response to ABA (Knight and others 2004). Xin and Browse (1998) identified an *Arabidopsis* mutant, eskimo1, that is constitutively freezing tolerant at warm temperatures, without changes in the expression of the components of the CBF regulon.

Considering the number of events ABA controls, for example, stress, dormancy, germination, stomatal closure, storage protein accumulation, and so on, it is difficult to conceive that only one ABA-inducible pathway exists. Recent evidence suggests that relative expression levels of two alleles of the same gene within the same cellular sample are regulated by variation in their cis-elements (Yan and others 2002). A similar scenario may also exist in the regulation of ABA-inducible genes. One of the ABA-inducible pathways uses a bZIP family of transcriptional factors that bind to the ABRE (Uno and others 2000). A second pathway utilizes MYC, and MYB proteins that interact with MYCR/MYBR elements (Abe and others 2003). MYC and MYB recognition sequences are found in the promoters of RD22 but not in the promoter region of LT178.

Dead Box RNA Helicase and ABA

In *Arabidopsis* the gene LOS4 (loss of osmotic responsiveness) is also involved in both chilling and freezing tolerance (Gong and others 2005). A recessive mutation, LOS4-1, impairs CBF cold-induced transcription that restricts chilling and freezing tolerance. LOS4 is thought to encode a putative DEAD box RNA helicase (Gong and others 2005). DEAD box proteins (dead represents the one letter amino acid code for the tetrapeptide, Asp-Glu-Ala-Asp) are members of the helicase superfamily of proteins, characterized by a common general function, an ATP-dependent nucleic acid unwinding. These proteins are implicated in alterations of RNA secondary structure, such as translocation initiation (elf-4A), pre-mRNA splicing, RNA chaperones, ribosome-biogenesis, RNA decay, organellar gene expansion, and nucelocytoplasmic transport (de la Cruz and others 1999; Tanner and Linder 2001; Lorsch 2002). Over 50 different DEAD box RNA helicase genes have been identified in the *Arabidopsis* genome. As stated above, the expression of many cold-acclimation–associated genes have in their proteins one or several copies of the DRE/CRT cis-element, which has the core sequence CCGAC (Yamaguchi-Shinozaki and Shinozaki 1994). CBF binds to this cis-element, which results in a transcriptional cascade of low temperature and stress-associated genes (Thomashow 1999). After analyzing 2 *Arabidopsis* mutants for DEAD box RNA helicases involved in mRNA nucleo-cytoplasmic transport, Gong and others (2005) suggested that CBF mRNA translocation can be a controlling point of cold acclimation. A mutant that exported mRNA from the nucleus quicker than the wild type cold-acclimated to a greater degree. This mutant was found to be hypersensitive to ABA.

Microarray transcript studies provide additional evidence that many of the genes upregulated in response to cold contain the CRT/DRE element and are not under the control of the CBF family (Fowler and Thomashow 2002). Also, constitutive expression of the CBF regulon does not result in cold acclimation of *Arabidopsis* plants (Haake and others 2002). The *Arabidopsis* HOSI (high expression of osmotic genes) protein is suggested to be a negative regulator of CBF genes (Lee and others 2001). HOS9 stress-associated proteins prevent or reduce cold acclimation. This gene is not directly involved in acclimation to freezing, but it is involved in acclimation to low-temperature growth. There are probably many other examples that demonstrate the cells must be able to metabolize normally to allow cold acclimation to proceed. Therefore, many genes upregulated in response to low temperature are associated with low-temperature growth acclimation and photoinhibition.

Summary

Freezing injury has been classified by Olien (1984) to be dependent on sub-zero temperatures. Death from prolonged exposure to temperatures lower than −10°C is the result of freeze-induced desiccation. Death at −10°C or at warmer temperatures is due to the mechanical and adhesive forces of the growing ice crystal. *Arabidopsis* would fall into the latter category, in contrast to hardy winter cereals or trees. Plants have evolved different strategies to cope with sub-zero temperatures, and therefore it should be expected that differences are equivalent at the molecular level. Fast cooling rates or extensive supercooling increases the free energy of freezing, resulting in the rapid formation of ice, which disrupts the cell walls of vascular tissue and sheers membranes (Olien 1967). This form of injury is largely due to the artificial freezing protocol, as few herbaceous plants supercool extensively in nature (Gusta and others 2004). Another anomaly introduced by the researcher occurs when plants growing in pots are transferred directly from 20°C to 4°C. The immediate drop in temperature decreases the hydraulic conductivity of the roots, which results in transient wilting, with a 10-fold decrease in water potential (Trischuk 2004). This is atypical of natural conditions, and the genes identified under these conditions may not be typical of those associated with cold acclimation. Although it has been demonstrated that there are a multitude of genes controlled by low temperature, it will probably be established that many of these are associated with cold shock, low-temperature growth acclimation, light acclimation, and so on. In studies employing growth chambers, the cold-acclimation reaction is driven continually by the low temperature selected by the researcher. This is a drastic contrast to natural conditions in which the temperatures fluctuate between acclimating and nonacclimating temperatures on a daily basis. Although the plant receives mixed messages, it still either continues to cold acclimate or maintain a level of freezing tolerance. It would be highly unlikely that ABA is involved in regulating all the genes associated with acclimation; however, it definitely regulates many important genes associated with acclimation.

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