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Plants in a cold climate

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Plants are able to survive prolonged exposure to sub-zero temperatures; this ability is enhanced by pre-exposure to low, but above-zero temperatures. This process, known as cold acclimation, is briefly reviewed from the perception of cold, through transduction of the low-temperature signal to functional analysis of cold-induced gene products. The stresses that freezing of apoplastic water imposes on plant cells is considered and what is understood about the mechanisms that plants use to combat those stresses discussed, with particular emphasis on the role of the extracellular matrix.

Keywords: cold; plant; freezing stress; cold acclimation; antifreeze protein

1. INTRODUCTION

Plants have adapted to living at freezing temperatures, enabling them to colonize high mountains and sub-arctic regions. The mechanisms that they use to survive sub-zero temperatures have been the subject of intense research for decades and recently modern technologies have elucidated some aspects of the molecular basis of plant frost tolerance. The purpose of this review is to introduce recent developments in our understanding of the adaptive mechanisms that plants employ to survive sub-zero conditions. Other articles in this issue provide more detailed analyses of the various aspects of adaptation to freezing temperatures.

Exposure of plants to low, but above zero, temperatures has long been known to enhance their subsequent tolerance of exposure to sub-zero temperatures. This process, known as cold acclimation, has been extensively studied, because analysis of the specific alterations associated with cold acclimation could reveal the molecular basis of freezing tolerance in plants (Steponkus 1984; Hughes & Dunn 1996; Thomashow 1999). The intrinsic academic interest of this is complemented by the potential biotechnological applications in broadening the geographical and seasonal locations at which crops can be cultivated.

In order for a plant to produce the appropriate adaptive responses, it needs (i) to perceive the low temperature; (ii) to transduce that signal to activate or repress expression of appropriate genes; and (iii) to utilize the genes to combat the diverse stresses that sub-zero temperatures impose on living cells. In addition, the low-temperature response has to be integrated with responses to other stress and developmental stimuli. In this context, it is important that many aspects of low-temperature stress are shared with other stresses, such as drought and salinity, and there is a commonality in the plant's adaptive mechanisms.

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2. PERCEPTION OF LOW TEMPERATURE

Exposure of plants to low temperature leads to a number of transient biochemical perturbations, any of which could act as perception points for initiation of the signalling cascades that elicit the stable developmental responses adapting them to the low-temperature environment. In addition to its direct effect on membrane fluidity, low temperature directly affects the stability of RNA and DNA secondary structures and the activity of enzymes, including those involved in fundamental processes such as transcription and translation, as well as intermediate metabolism and photosynthesis. These perturbations produce secondary effects, such as energy imbalance, that may be exploited as part of the plant's low-temperature detection apparatus (Huner *et al.* 1998). Low temperature also imposes a dehydrative stress, by lowering water absorption by the root and water transport in the shoot.

A direct and early effect of low temperature on living cells is a decrease in membrane fluidity (Levitt 1980) and it has long been speculated that the perception of low temperature could be based on alterations in the physical state of the bilayer. There is some preliminary evidence to support this hypothesis. Murata & Los (1997) extended the observations made in *Synechocystis*, that rigidification of membranes could induce expression of cold-regulated genes (Vigh *et al.* 1993). More recently, Orvar *et al.* (2000) showed that cold-induced accumulation of a low-temperature responsive gene in alfalfa could be prevented by fluidization of the membrane with benzyl alcohol and, conversely, rigidification of the membrane by dimethylsulphoxide led to induction of the gene at non-acclimating temperatures.

A number of observations indicate that plant cells sense the rate of temperature change (dT/dt) rather than the absolute temperature. This hypothesis was expanded by Minorsky (1989) and Minorsky & Spanswick (1989) who showed that at temperatures between 22 and 16 °C rapid cooling rates elicited strong depolarizations in cucumber seedlings, whereas slow cooling rates did not. Further evidence was supplied by Plieth *et al.* (1999), who monitored cold-induced cytosolic calcium transients in *Arabidopsis*

and showed that the magnitude of calcium influx correlated with the rate of temperature drop.

An early event in a plant's response to low temperature is an influx of calcium from the apoplast into the cytosol (Knight *et al.* 1991, 1996; Monroy *et al.* 1993; Monroy & Dhindsa 1995). It has been suggested that calcium channels located in the plasma membrane may act as one of the sensors of temperature fall, and Ding & Pickard (1993) have shown that mechano-sensitive calcium channels are activated by cold. More recent data suggest that cold-induced calcium transients may occur downstream of membrane rigidification and cytoskeletal reorganization (Orvar *et al.* 2000; Sangwan *et al.* 2001).

Although preliminary evidence suggests that the physical state of the membrane may be important in low-temperature perception, the molecules that sense these changes in plants have not yet been identified. Two-component sensor–response regulator systems are used by plants to sense plant hormones, such as ethylene (Chang & Shockey 1999), and have been implicated in the detection of osmotic stress (Urao *et al.* 1999, 2000). Expression of two-component response regulator-like proteins has been shown to be induced by low temperature in *Arabidopsis* (Urao *et al.* 1998), and two histidine kinases and a response regulator have been identified in *Synechocystis* that modulate low-temperature regulation of some, but not all, cold-responsive genes (Suzuki *et al.* 2000, 2001). *Bacillus subtilis* has also been shown to employ a two-component sensor–response regulator as a thermosensor (Aguilar 2001). It is possible that the low-temperature detection systems of plants could involve one or more two-component response regulator proteins.

Plants do not experience low-temperature stress in isolation from other environmental insults and, although many of the genes that are responsive to low temperature are also regulated by other abiotic stresses, some respond specifically to cold (Hughes & Dunn 1996). Perception of stress is one of the means by which specificity can be encoded into the signal transduction network that leads to adaptation of the plant's physiology to a particular environment (Knight & Knight 2001). Although alterations in the physical state of the bilayer have received the most attention as a point of low-temperature perception, it is probable that other direct effects of cold on cellular metabolism are also perceived and used to integrate the plant's response as has been shown in prokaryotic systems (Hurme *et al.* 1997; Hurme & Rhen 1998).

3. SIGNAL TRANSDUCTION

Freezing tolerance is a multigenic trait reflecting the multifaceted complexity of the stress imposed on plant cells by sub-zero temperatures. Altering the expression of an individual gene that addresses a single aspect of freezing stress is therefore unlikely to significantly alter the freezing tolerance of the whole plant. In consequence, the alterations associated with cold acclimation must involve the coordinate induction or repression of multiple genes. Screening *Arabidopsis* mutants for lines that are either constitutively freeze tolerant (Xin & Browse 1998) or freeze susceptible following cold acclimation (Warren *et al.* 1996) therefore usually uncovers lesions in signalling where expression of a 'regulon' of genes has been affected. Equally, mutants identified in screens specifically designed to isolate genes involved in abiotic stress signalling (Ishitani *et al.* 1997*b*) often display altered freeze tolerance (Ishitani *et al.* 1998; Lee *et al.* 1999). Manipulation of signalling factors such as protein kinases, phosphatases or transcription factors offers a practical means to engineer the stress tolerance of crop species.

In addition to enhancing freeze tolerance, prolonged exposure to low temperature reduces the time to flowering in *Arabidopsis*, a process known as vernalization. In wheat, although freezing tolerance and vernalization are closely linked, they appear to be controlled by different genes (Sutka 2001). Until recently, the genes that control vernalization and freezing tolerance in *Arabidopsis* appeared to be independent (Chandler *et al.* 1996) but recently a signalling mutant has been characterized that affects both responses (Ishitani *et al.* 1998; Lee *et al.* 2001).

Low-temperature stimuli have to be integrated with developmental status and responses to other biotic and abiotic stresses. The increased freezing tolerance afforded by exposure to other abiotic stresses, such as drought or exogenous application of ABA, has been demonstrated repeatedly. This is reflected in the genes that are stress regulated, many of which are responsive to multiple biotic and abiotic stimuli. However, some genes are specifically regulated by low temperature (reviewed in Hughes & Dunn 1996; Thomashow 1999); genes that respond to multiple abiotic stresses, such as RD29A (Xiong *et al.* 1999), as well as those that are specifically responsive to cold and not other stress stimuli, such as the carrot AFP gene (Worrall *et al.* 2002), are both affected in their low-temperature response when this is combined with exposure to other abiotic stresses. Possible 'nodes' at which this cross-talk between abiotic stress signals could occur have been discussed in a number of excellent reviews (Shinozaki & Yamaguchi-Shinozaki 2000; Knight & Knight 2001; Xiong & Zhu 2001). The purpose of this section is to introduce the issues raised by transduction of low-temperature signals, rather than to provide a comprehensive review.

(a) *Early events*

An early event in a plant cell's response to low temperature is a transient elevation of intracellular calcium (Knight *et al.* 1991) initiated by calcium influx through the plasma membrane and release from the vacuole (Knight *et al.* 1996). Elevation of intracellular calcium has been shown to be sufficient and necessary to promote the expression of low-temperature responsive genes in alfalfa (Monroy & Dhindsa 1995) and *Arabidopsis* (Knight *et al.* 1996). Orvar *et al.* (2000) have published data suggesting that this influx is regulated by reorganization of the cytoskeleton triggered by rigidification of the membrane (Orvar *et al.* 2000; Sangwan *et al.* 2001).

Elevation of intracellular calcium is characteristic of many abiotic and biotic stresses but cellular responses are specific to the individual stress. The means by which different responses can be regulated by the same messenger have recently been reviewed (McAinsh & Hetherington 1998; Sanders *et al.* 1999). They include cellular recognition of the frequency, duration, amplitude or subcellular localization of the calcium elevation. The calcium signature of a cell responding to low temperature, and its

response to that signature, also depend on both the cell type and its stress history. For example, calcium transients in *Arabidopsis* shoot tissues responding to cold shock are significantly higher than those found in root tissue (Kiegle *et al.* 2000) and the cold-induced calcium signature of stress-adapted plants is different from that in non-adapted plants (Knight *et al.* 1996, 1998; Knight & Knight 2000). On a shorter time-scale, calcium transients are subject to attenuation on repeated exposure to identical cooling regimes and sensitization by lower absolute temperatures (Plieth 1999; Plieth *et al.* 1999).

Calcium transients can be transduced by a number of intracellular sensors such as calmodulin, CDPKs and calcium-sensitive PPs. There is evidence for involvement of all these sensors in the transduction of abiotic stress stimuli but the specific members of each family that are used to integrate low-temperature signal(s) into the plant's response have not yet been identified. Calmodulin has been implicated in response to numerous abiotic stresses, including low temperature (Braam & Davis 1990; Snedden & Fromm 2001).

The CDPK family of protein kinases is unique to plants and there are approximately 40 in the *Arabidopsis* genome (Harmon *et al.* 2000): two of these have been implicated in the transduction of drought- and salt-stress signals (Urao *et al.* 1994; Sheen 1996). An abundance of transcript for the rice CDPK, OsCDPK7, has been shown to increase following exposure to low temperature. However, overexpression of OsCDPK7 did not affect cold-regulated gene expression although it did increase seedling chilling tolerance and expression of salt- and drought-regulated genes in rice (Saijo *et al.* 2000). The CDPKs specifically involved in transduction of low-temperature stimuli remain to be identified.

The PP families 2B and 2C (calcineurin-B-like) are both calcium dependent: a role for proteins related to the calcineurin B proteins has been demonstrated in salt-stress signalling (Liu & Zhu 1998; Zhu *et al.* 1998) and transduction of the plant-stress hormone ABA (Leung & Giraudat 1998; Gosti *et al.* 1999) but not yet for low temperature. In contrast, inhibition of the calcium-independent PPs, PP2A, led to the expression of cold-regulated genes at ambient temperatures, and cold-triggered calcium-dependent inhibition of PP2A has been demonstrated in alfalfa (Monroy *et al.* 1998). It has been suggested that PP2A could physically associate with CDPKs to regulate their activity, as has been demonstrated in mammalian cells (Xiong & Zhu 2001).

A signalling module that is widely used throughout eukaryotes is the MAPK cascade. The core of the MAPK module consists of three kinases, MAPK, MAPKK and MAPKKK. Upon activation of the MAPKKK, these proteins sequentially phosphorylate each other yielding an ultrasensitive on/off switch-type mechanism (Robinson & Cobb 1997). MAPK-signalling modules offer a means to introduce specificity into a signalling network, via both the signal and substrate specificity of the proteins that activate individual MAPKKKs and the specificity and physical association of specific MAPKKs and MAPKs often via scaffold proteins.

MAPK cascades have been implicated in a number of signal transduction pathways in plants including responses to environmental stress and ABA. Jonak *et al.* (1996) demonstrated that a MAPK was rapidly activated by cold stress and expression of an *Arabidopsis* MAPKKK, and a MAPK gene, ATMPK3, was shown to be induced by a number of different stresses, including low temperature (Mizoguchi *et al.* 1996). Two *Arabidopsis* MAPKs have demonstrated rapid activation in response to cold, as well as other abiotic stresses, but the upstream components of the cascade have not been identified (Ichimura *et al.* 2000). Although overexpression in *Arabidopsis* of NPK1, a MAPKKK that is involved in H_2O_2 signalling, increased tolerance to freezing as well as other abiotic stresses, the expression of RD29A, a gene that is known to be stress responsive, was unaffected (Kovtun *et al.* 2000). These data suggest that the MAPK-pathway-transducing signals of oxidative stress are independent from that of other abiotic stresses; since oxidative stress is a component of most abiotic stresses, it has been suggested that reactive oxygen species could potentiate stress signalling (Xiong & Zhu 2001), as implied by the increased freezing tolerance of the transgenic NPK1 overexpressors. Although it is likely that MAPK cascades are involved in transduction of low-temperature signals, as with the CDPKs, the specific enzymes employed have not been identified.

(b) The *CBF*/DREB 'regulon'

The promoters of many ABA-independent cold- and drought-induced proteins contain one or more copies of a *cis*-acting element with the core sequence CCGAC known as the CRT (Baker *et al.* 1994) or DRE (Yamaguchi-Shinozaki & Shinozaki 1994). This element has been shown to bind a group of three similar cold-induced transcriptional activators, known as either CBF1–3 (Stockinger *et al.* 1997) or DREB1A–C. The CRT/DRE also binds drought-inducible transcriptional activators DREB2A–B that are structurally unrelated to the CBF/DREB1 group. Overexpression of the *CBF*/*DREB1* gene in *Arabidopsis* led to enhanced frost tolerance and the induction of known cold-responsive genes (Jaglo-Ottosen *et al.* 1998; Liu *et al.* 1998) as well as mimicking many of the other biochemical changes associated with cold acclimation (Gilmour *et al.* 2000). The growth-retarding effect of this group of transcription factors was minimized by placing the genes under the control of a stress-responsive promoter (Kasuga *et al.* 1999).

The *CBF*/*DREB1* family of transcription factors is arranged in a tandem array on chromosome 4 of the *Arabidopsis* genome (Gilmour *et al.* 1998) and is induced transiently in response to cold. The promoters of the *CBF*/*DREB1* transcription factors do not contain the DRE element and do not appear to autoregulate their own expression. Thomashow (1999) has proposed the presence of an ICE regulatory protein that may be activated upon cold shock, leading to induction of the transcription factors.

A further component of the signalling cascade(s) that controls CBF/DREB-regulated gene expression has been characterized genetically. Warren *et al.* (1996) identified a series of mutants that were deficient in cold-induced freezing tolerance. One of these, *sfr6*, was shown to specifically attenuate cold-induced transcription of genes regulated by the CBF/DREB1 group of transcription factors (Knight *et al.* 1999). Cold-induced transcription of genes whose promoters do not contain the DRE repeat,

such as *AtP5CS1* and *CBF/DREB1*, was unaffected by the mutation, and cold-induced calcium transients were also indistinguishable from wild-type. The *sfr6* mutation was therefore located between cold-triggered calcium elevation and transcriptional activation of genes induced by CBF/DREB1 (Knight *et al.* 1999).

Interactions between low temperature and other abiotic stresses are known to affect cold-regulated gene expression. For instance, pre-exposure of plants to NaCl delayed the low-temperature induction of a reporter gene fused to the promoter of RD29A, a gene that is transcriptionally regulated by numerous abiotic stresses as well as by ABA (Xiong *et al.* 1999). Some of these interactions may be mediated by the cold-inducible and drought-inducible transcription factors that interact with DRE elements. However, others will be mediated by other nodes within the signal transduction network (Knight & Knight 2001).

(c) *Parallel pathways to low-temperature-regulated genes?*

Some *Arabidopsis* genes that are cold inducible do not contain the CRT/DRE element in their promoters, for instance alcohol dehydrogenase and *AtP5CS1*, a key enzyme in proline accumulation. Equally, two cold-inducible genes that are thought to be critical in controlling the level of sucrose in plants, sucrose phosphate synthase and sucrose synthase were not upregulated in plants overexpressing CBF3 (Gilmour *et al.* 2000). Xin & Browse (2000) have suggested that cold-regulated gene expression in plants could consist of several parallel pathways that activate overlapping 'suites' of genes important in freezing tolerance.

The *esk1* mutant of *Arabidopsis* appears to define a low-temperature signalling pathway that is independent of the CBF regulon. This recessive mutant, which shows constitutive freezing tolerance in the absence of cold acclimation, has elevated levels of proline arising from both constitutively elevated *P5CS1* expression and a block of proline-dependent induction of the proline oxidase gene. Interestingly, expression of the *cor* genes that are controlled by the CBF/DREB1 transcription factors is not constitutively elevated, although these genes are still responsive to low temperature in the mutant (Xin & Browse 1998).

The regulation of expression of P5CS, a key enzyme in proline biosynthesis, provides an interesting example of redundancy in low-temperature regulation of gene expression. There are two copies of the *P5CS1* in the *Arabidopsis* genome (Strizhov *et al.* 1997), one of which has DRE elements upstream of the coding sequence (*AtP5CS2*) and one of which does not (*AtP5CS1*) but expression of both genes is upregulated by low temperature. Expression of *AtP5CS2* appears to be under the control of the CBF/DREB group of transcription factors since its expression is increased in plants constitutively expressing *CBF1/DREB1B* (Gilmour *et al.* 2000). In contrast, expression of *AtP5CS1* is not elevated in plants overexpressing *CBF3/DREB1A* (Kasuga *et al.* 1999). It is possible that this difference could arise from the different members of the CBF/DREB1 family used in these experiments; however, *AtP5CS1* is cold inducible in *sfr6*, a mutant that has a lesion in the CBF signalling pathway (Knight *et al.* 1999), suggesting that its regulation by low temperature is independent of the CBF/DREB pathway.

Light is also a critical signal in cold acclimation. Shortening day length is associated with the onset of autumn and cold acclimation. Plants compromised in their perception of day length are also compromised in cold acclimation (Olsen *et al.* 1997). High light and low temperature both result in induction of genes such as *Wcs19* and a compact morphology in rye (Huner *et al.* 1998). Light is necessary for cold-induced freezing tolerance in *Arabidopsis* (Wanner & Juntilla 1999) and high light enhances cold-induced freezing tolerance in rye (Huner *et al.* 1998). Light does not directly control low-temperature induction of many cold-regulated genes, but common elements of low temperature and light stress, such as production of reactive oxygen species and photosystem II excitation pressure, influence the low-temperature signal transduction mechanisms and thus the physiological responses to low temperature.

Many cold-regulated genes are members of small multigene families that show organ-specific expression and respond differentially to low temperature (Pearce *et al.* 1998). The regulation of *P5CS* by low temperature may represent a simple example of the alternative pathways that different tissues employ in cold-regulated signal transduction. The presence of parallel pathways may also offer flexibility in the cross-talk between abiotic or other signalling networks that enable alternative modifications of metabolism in a manner appropriate to combined stresses.

(d) *Signalling factors involved in both vernalization and cold acclimation*

Ishitani *et al.* (1997*a*,*b*) isolated two recessive signalling mutants, *hos1* and *hos2*, which showed hypersensitivity specifically in their response to low temperature and not in their response to other abiotic stresses. Both lesions affected CBF/DREB-dependent and CBF/DREB-independent pathways but the *hos2* mutation did not affect vernalization (Lee *et al.* 1999), whereas the *hos1* mutant showed constitutive vernalization (Ishitani *et al.* 1998). *HOS1* is the first gene that has been shown to affect both cold acclimation and vernalization and thus represents a central player in the transduction of low-temperature stimuli in plants.

Lee *et al.* (2001) have elegantly demonstrated the characteristics of the *HOS1* gene product that are likely to be critical to its cellular function. The *HOS1* gene encodes a RING finger protein that displays cold-regulated nuclear-cytoplasmic partitioning (Lee *et al.* 2001) and thus represents a connection between cold-induced cytoplasmic events and nuclear gene transcription. *HOS1* appears to negatively regulate transcription of *CBF/DREB1* and thus transcription of the genes that these control as well as other cold-induced genes whose promoters do not contain the CRT/DRE element. *HOS1* transcript abundance dipped transiently 10 min after exposure to low temperature and returned to normal levels within 1 h. This raises the possibility that it may interact with the putative ICE regulator of CBF expression since induction of CBF transcript is detectable *ca*. 30 min after a low-temperature stimulus.

Other kinetic aspects of *HOS1* transcription and nuclear–cytoplasmic partitioning are interesting. The

HOS1 transcript disappeared between 24 and 48 h after transfer to cold conditions and the cold-induced accumulation of the HOS1–GFP fusion protein in the nucleus persisted over days but disappeared within 12 h on returning to normal growth temperatures. Thus *HOS1* represents a signalling element whose kinetics, both at the transcript and protein levels, appear to relate to the persistent effect of cold on gene expression under continued exposure to low temperature and its rapid reversal on return to normal conditions.

HOS1 is a RING finger protein: these have recently been shown to be mediators of E3 ubiquitin ligase activity (Freemont 2000; Jackson *et al.* 2000) for both heterologous substrates and themselves. The stability of critical regulatory proteins, such as the HY5 transcription factor that mediates light-activated gene expression, appears to be dynamically regulated by RING finger proteins (Osterlund *et al.* 2000). Although no biochemical activity has yet been demonstrated for *HOS1*, it seems likely that at least part of its role in low-temperature signal transduction may be mediated by regulating the stability of crucial signalling proteins.

(e) *Post-transcriptional regulation of gene expression*

Whilst regulation of gene expression at the transcriptional level has received most attention, it can occur at other levels: for instance the processing, transport and stability of transcript, the rate of translation and the stability of the gene product all contribute to regulation.

A number of genes whose transcript abundance is affected by low temperature appear to be regulated post-transcriptionally in a range of monocot and dicot species (Hajela *et al*. 1990; Wolfraim & Dhindsa 1993; Dunn *et al.* 1994). Phillips *et al.* (1997) concluded that, on the basis of pharmacological inhibition of protein and RNA synthesis, the transcript of a barley cold-regulated gene, *blt14.0*, was actively stabilized by a protein factor.

A group of proteins that has received some attention in terms of low-temperature regulation of gene expression are the glycine-rich proteins that contain the RRM (reviewed in Alba & Pages 1998). These are small proteins consisting of two domains, a C-terminal glycine- and arginine-rich region and an N-terminal RRM. The RRM is found in proteins that bind pre-mRNA, mRNA, pre-rRNA, small nuclear RNAs and chloroplast or mitochondrial RNAs. Plant glycine-rich RRMs that are cold induced are present in many species including *Arabidopsis* (Carpenter *et al.* 1994), leafy spurge (Horvath & Olson 1998), potato (Baudo *et al.* 1999) and barley (Dunn *et al.* 1996).

In addition to enhanced expression under low-temperature conditions, expression of plant glycine-rich RRM proteins has been shown to be regulated developmentally as well as by exposure to numerous biotic (Naqvi *et al.* 1998; O'Hara *et al.* 1998) and abiotic (Gomez *et al.* 1988; Van Nocker & Vierstra 1993; Horvath & Olson 1998) stresses. Their role at low temperature is particularly interesting: the expression of structurally related proteins from organisms as diverse as cyanobacteria (Sato 1994) and mammals (Nishiyama *et al.* 1997) is subject to low-temperature control. Although the plant, mammalian and cyanobacterial proteins are structurally related, they do not appear to have evolved from a common ancestor and similarly their regulation by cold is likely to have evolved independently, providing an interesting example of convergent evolution in low-temperature responses (Maruyama *et al.* 1999). The molecular function of this class of proteins at low temperature is still speculative, although the sub-cellular localization of different members suggests a role in rRNA metbolism or pre-mRNA processing (Alba & Pages 1998).

Gene regulation at the translational level allows rapid alteration of the abundance of gene product as compared with transcriptional regulation, and can be executed via controlled seqestration of mRNAs on mRNA ribonucleoproteins, recruitment of mRNA to the ribosome, elongation of the polypeptide chain or polypeptide chain release (reviewed in Bailey-Serres 1999). Although stresses such as heat shock and anoxia have been shown to affect the selective translation of stress-related mRNAs and impair the translation of normal cellular transcripts (Bailey-Serres 1999), there are few studies on low-temperature regulation of gene expression at the level of translation in plants. The expression of the components of the translational apparatus is regulated by low temperature (Dunn *et al.* 1993). Horiguchi *et al.* (2000) showed that levels of a cold-induced fatty acid desaturase were controlled at the level of translation and Mastrangelo *et al.* (2000) demonstrated that the levels of a putative amino-acid selective transporter were controlled at the level of translation as well as post-transcriptionally.

An interesting observation, in terms of the translational control of gene expression, is the induction of a ribosomal S6 kinase (Mizoguchi *et al.* 1995, 1996) and TAP46 (Harris *et al.* 1999) by low temperature. TAP46 shows sequence similarity with yeast TAP42 and the mammalian α4-protein family. These proteins are involved in the activation of a ribosomal S6 kinase that inactivates a translation initiation inhibitor, 4E-BP1. This results in increased mRNA translation through enhanced activity of the translation initiation factor eIF-4E. Interestingly, TAP46 has been shown to interact *in vivo* with PP2A (Harris *et al.* 1999), an enzyme that is thought to be involved in low-temperature signal transduction in plants (Monroy *et al.* 1998).

(f) *The role of hormones in cold acclimation*

Another pathway that affects freezing tolerance is that mediated by ABA. The levels of the phytohormone ABA are transiently elevated in response to low temperature (Chen *et al.* 1983), and exogenous application of ABA at non-acclimating temperatures can enhance freezing tolerance (Lang *et al.* 1994) as well as inducing many of the genes that respond to low temperature (Heino *et al.* 1990; Gilmour & Thomashow 1991). Mutants that are deficient in the biosynthesis of ABA (*aba* mutants) appear less able to cold acclimate and this deficiency can be rescued by exogenous application of ABA (Heino *et al.* 1990; Gilmour & Thomashow 1991; Llorente *et al.* 2000). By contrast, cold-induced freezing tolerance is unaffected in *Arabidopsis* mutants that are insensitive to ABA (*abi* mutants) (Gilmour & Thomashow 1991). Cold-inducible genes are still responsive to low temperature in both the *aba* and the *abi* mutants. These apparently conflicting data may be reconciled by the hypothesis that different ABA receptors mediate transduction of ABA signals for the

genes involved in cold acclimation from those used in other responses to ABA.

Induction of many genes by low temperature is ABA-independent (Gilmour & Thomashow 1991; Nordin *et al.* 1991), and ABA-dependent induction is mediated by *cis*-acting elements different from those mediating the cold response (Yamaguchi-Shinozaki & Shinozaki 1994). Consequently, the importance of ABA in cold acclimation has been questioned (Shinozaki & Yamaguchi-Shinozaki 2000). However, it is known that cold stress interacts with ABA-dependent pathways (Ishitani *et al.* 1997*a*,*b*) and the *sfr6* mutation affects ABA-dependent gene induction as well as CBF/DREB1 and DREB2-dependent induction (Knight *et al.* 1999). It is possible that the cold-regulated gene expression that is affected in the *aba* mutants, altering their susceptibility to low temperature, represents another parallel pathway in the low-temperature regulatory network.

The role of other plant hormones in cold acclimation is less well studied than that of ABA, but it is possible that they influence the process, both through modulation of development and through temperature dependence of their perception, transduction and response. Gibberellin-response mutants exhibit altered responses to low temperature: for example, the slender mutant of barley does not show the normal low-temperature inhibition of elongation growth (reviewed in Schunmann *et al.* 1994). Biosynthesis of the plant hormone auxin is affected by temperature in *Arabidopsis* (Gray *et al.* 1998) and cellular sensitivity to gibberellin is affected by cold (Hisamatsu & Koshioka 2000). Alterations in the status of these hormones may affect cold acclimation; for example, a deficiency in cold acclimation was detected in aspen that had altered daylength responses resulting from transformation with an oat phytochrome, and had an associated change in abundance of gibberellins and auxin (Olsen *et al.* 1997).

4. STRESSES IMPOSED BY SUB-ZERO TEMPERATURES

Under the slow cooling regimes found in nature, ice usually crystallizes in the apoplast because of the lower solute content and the presence of nucleators in this compartment. When water freezes in the apoplast, multiple stresses are imposed on plant cells; these include:

(i) direct thermal effects on bilayers, proteins and other macromolecules;
(ii) chemical stresses imposed by withdrawal of water leading to an increased concentration of solutes, especially ions, increased molecular crowding, dehydration of membranes and macromolecules;
(iii) mechanical stresses imposed on membranes by alterations of cell volume, on the extracellular matrix by growing ice crystals or as dissolved gases that are concentrated in the unfrozen solution and return to the gas phase;
(iv) electrical perturbations due to potential differences produced at a rapidly growing ice–water interface.

The primary manifestation of injury is observed at the plasma membrane (Steponkus 1984). This is reflected in *in vitro* assays for freezing damage, which are almost exclusively based on measurements of the integrity of the plasma membrane. A functionally intact plasma membrane prevents incursions of apoplastic ice crystals into the cytosol but the chemical potential of extracellular ice draws water out of the cell. This process imposes a dehydrative stress on cellular membranes and a problem of sufficient surface area to accommodate the reduction of cell volume associated with the loss of intracellular water. The permeability of the plasma membrane (and hence the tonoplast) to water is also critical: the intracellular solution will be supercooled until it has reached equilibrium with the chemical potential of the extracellular ice, and it will therefore be predisposed to the formation of ice; crystallization of intracellular ice is usually considered to be lethal.

Three forms of injury to the plasma membrane are observed on thawing cells that have been frozen: expansion-induced lysis, loss of osmotic responsiveness and altered osmotic behaviour (Steponkus 1984). These different types of injury reflect the different stresses that are imposed when plant cells are frozen.

(a) *Mechanical stresses*

When extracellular ice freezes, the intracellular volume decreases as water is drawn out of the cell. Because the intrinsic elasticity of lipid bilayers is only 2–3% (Wolfe *et al.* 1985), membrane must either be deleted, for instance by vesiculation of the plasma membrane, or conserved by invaginations or extrusions of the plasma membrane to increase the surface area : volume ratio. In the former scenario, material deleted from the membrane must be reincorporated, or new material must be added, to enable the cell to regain its former volume when the ice thaws. Expansion-induced lysis occurs if the cells cannot maintain plasma membrane area or, alternatively, reincorporate material sufficiently rapidly. Expansion-induced lysis is a commonly observed form of injury observed in non-acclimated cells of rye (Uemura & Steponkus 1989), oat (Webb *et al.* 1994) and *Arabidopsis* (Steponkus *et al.* 1998), at temperatures above -4°C.

The cell wall of plant cells imposes an additional mechanical stress on the plasmalemma and connected internal membranes. The membrane systems of plant cells are connected to the extracellular matrix (reviewed in Kohorn 2000) and, in intact tissues, cells are connected to each other via the extracellular matrix and symplastically through plasmodesmata. These connections will impose a mechanical strain on the internal membrane systems whether the cell plasmolyses or undergoes cytorrhysis in response to extracellular ice. Although the temperature at which 50% of cells die (LT_{50}) is similar in intact rye mesophyll cells and in protoplasts derived from them (reviewed in Steponkus 1984), this is not true of all cell types. Tao *et al.* (1983) and Murai & Yoshida (1998) have shown that protoplasts from cultured potato cells and Jerusalem artichoke, respectively, have significantly greater freeze tolerance than the intact cells or tissues from which they are derived, suggesting that the cell wall is a limiting factor in freeze tolerance of these cells.

Another source of freeze-induced mechanical damage is cavitation. In intact cold-hardened tissues, some cell types, such as the xylem ray parenchyma cells of many

tree species, do not dehydrate in response to extracellular ice but supercool. Significant negative pressures can develop in these cells (Rajeshakar & Burke 1996), and the consequent formation of vapour bubbles causes damage and subsequent collapse on return to normal pressures (Weiser & Wallner 1988).

(b) *Dehydrative stresses*

The dehydrating effect of extracellular ice dominates the biophysical effects of sub-zero temperature on cell membranes. There is relatively little evidence that the direct effects of temperature on membranes are responsible for freeze-related damage, but a significant body of data suggests that the dehydrative effect of ice crystallization, combined with low temperature, are responsible for the injury (Steponkus 1984; Wolfe & Bryant 1999). The freezing temperature directly determines the chemical potential of ice and thus the dehydrative pressure that extracellular ice exerts on the intracellular compartments. The biophysical effects of freezing and dehydration on cell membranes have been the subject of an accessible review (Wolfe & Bryant 1999) and are therefore only dealt with briefly here.

First, under conditions of low hydration, the temperature at which bilayers change from the fluid to the gel phase (T_m) is increased. Second, intrinsic membrane proteins can become preferentially sequestered in domains that are relatively highly hydrated, because they have greater hydration than the bilayer lipids that surround them. Third, the phospho- or glycolipids with more hydrated head groups can separate from those with less hydrated head groups into phases that are enriched for one or the other. Finally, at very low hydrations, the lateral stress on the plane of the membrane can result in the transition of membrane lipids from the lamellar to the HexII phase. When membranes are closely apposed as in a dehydrated cell, formation of the HexII phase in one membrane will be propagated into other adjacent membranes, compromising cellular compartmentalization. Any of the first three biophysical effects may alter the semi-permeable characteristics of cellular membranes when cells are returned to above zero temperatures, leading to altered osmotic responsiveness. The loss of compartmentalization, resulting from the formation of HexII phases between membranes defining different sub-cellular compartments, leads to a loss of osmotic responsiveness.

The visible manifestations of freeze-induced injury at the plasma membrane, detectable at the level of the electron microscope, are the occurrence of lateral phase separations, inverse HexII phase in closely apposed cellular membranes, and fracture jump lesions (reviewed in Steponkus 1984). The formation of the HexII phase is thought to be the major cause of cell death in non-acclimated plants but is effectively prevented in acclimated cells.

5. PLANT STRATEGIES FOR SURVIVAL OF SUB-ZERO TEMPERATURES

Plants that survive in a vegetative state through the winter can either prevent the crystallization of ice within their tissues (freeze avoidance) or allow ice to crystallize in the apoplast (freeze tolerance). Freeze avoidance involves supercooling and hence prevention of incursion ice into the apoplast. Given the widespread presence of nucleators in the environment and the relative openness of the apoplastic compartment in aerial plant tissues, it is only a practical strategy at the whole plant level when exposure to sub-zero temperature is relatively brief. However, some specialized cell types and organs do use supercooling as a strategy to overwinter: the xylem ray parenchyma cells and floral primordia of many trees supercool to *ca.* −40 °C (Quamme 1974; George & Burke 1977).

Common features of alterations to cell biochemistry associated with cold acclimation include:

(i) the accumulation of compatible (i.e. non-toxic) solutes;
(ii) alterations in membrane lipid composition (reviewed in Uemura & Steponkus 1999);
(iii) increased antioxidant activity (Tao *et al.* 1998);
(iv) altered composition and increased strength and thickness of cell walls (Waddell & Wallner 1984; Wallner *et al.* 1986; Rajashakar & Lafta 1996; Kozbial *et al.* 1998; Kubacka-Zebalska & Kacperska 1999; Stefanowska *et al.* 1999);
(v) altered patterns of gene expression including those encoding proteins that remain soluble upon boiling (Guy 1990; Thomashow 1999).

As it is rarely clear which particular aspect of freezing stress is responsible for cell death at a particular temperature, it is equally difficult to define which cold-induced alteration addresses the lethal stress. The absence of effect of deletion or overexpression of a gene on freezing tolerance does not indicate that the gene has no role in tolerance, merely that it does not address the lethal stress at that particular stage of acclimation, and under the particular conditions that were used for assay.

This section introduces some aspects of the changes associated with cold acclimation, together with current hypotheses of how these may function in plant survival of sub-zero temperatures.

(a) *The role of membrane lipids in freezing tolerance*

Cold-induced alterations to the lipid composition of cellular membranes, particularly the degree of non-saturation, have an important role in chilling tolerance (reviewed in Nishida & Murata 1996), and it is not easy to separate these effects from their role in freezing tolerance. Cold acclimation results in alterations in the proportions of almost all membrane lipid species and these are associated with specific changes in the nature of freeze-induced lesions (Uemura & Steponkus 1994; Webb *et al.* 1994).

Cold acclimation results in alterations to cellular membranes that enable the cell to resist expansion-induced lysis (Webb *et al.* 1994). Surface area can be conserved in plasmolysing cells by the production of Hechtian strands, long tubes of plasma membrane joining the contracted cell to the wall, that yield a large surface area : volume ratio (reviewed in Oparka *et al.* 1996). An increase in the numbers and strength of Hechtian strands following cold hardening has been observed (Scarth 1941; Johnson-Flanagan & Singh 1986; Buer *et al.* 2000). The mechanisms

by which membrane is deleted and reincorporated are also altered following cold acclimation in rye and wheat (reviewed in Steponkus 1984; Singh & Johnson-Flanagan 1987).

The lipid composition of cellular membranes affects the cells' propensity to undergo expansion-induced lysis. Plasma membrane lipids from acclimated cells and di-unsaturated phospholipids have been shown to alter the manner in which both protoplasts and liposomes vesiculate in response to freezing. This has been shown to correlate with survival of expansion-induced lysis (Steponkus *et al.* 1988*a*,*b*; Steponkus & Lynch 1989; Uemura & Steponkus 1994). A proliferation of vesicular structures and of endomembranes is observed early in the process of cold acclimation in many species, including *Arabidopsis* (Ristic & Ashworth 1993), but we are not aware of any reports on membrane ultrastructure in constitutively frost-tolerant mutants or transgenics.

The cold-induced reduction in the injury arising from lamellar to HexII transition is also associated with alterations to the lipid composition of membranes, including the degree of unsaturation of phospho- and glycolipids and the relative proportions of sterols, cerebrosides and phospholipids, particularly phosphatidylcholine (Uemura & Steponkus 1994). The introduction of unsaturated phosphatidylcholine species into the plasma membrane of protoplasts derived from non-acclimated rye leaves precluded the formation of HexII phase in the plasma membrane (Sugawara & Steponkus 1990). The proportion of acylated sterylglucosides and free sterols in a bilayer affects its intrinsic curvature, and hence its propensity to form the HexII phase. These proportions are altered in cold-acclimated cells and have been shown to correlate with the occurrence of this form of injury (Uemura & Steponkus 1994). Cerebrosides are the least hydrated species in the plasma membrane and therefore promote lipid demixing and reduce the overall hydration force that separates bilayers in dehydrated cells. Again, the phospholipid : cerebroside ratio has been shown to increase on cold acclimation and the ratio has been shown to correlate with a reduced incidence of the HexII phase (Uemura & Steponkus 1994).

Fracture jump lesions, rather than HexII phase, are associated with the loss of osmotic responsiveness in cold-acclimated cells (Webb *et al.* 1994) and the temperature at which these occur decreases progressively with the duration of cold acclimation (Fujikawa & Steponkus 1990; Webb *et al.* 1994). Fracture jump lesions may arise from interlamellar attachments between the plasma membrane and closely apposed endomembranes, or by interdigitation of lipids that have undergone a liquid-crystalline to gel phase transition. In either scenario, the biophysical properties of the increased phosphatidylcholine : cerebroside ratio that occur in cold-acclimated tissue are predicted to reduce the occurrence of this type of lesion (Uemara & Steponkus 1994).

(b) *Compatible solutes*

Cold acclimation is associated with the accumulation of a range of low-molecular-weight organic solutes including proline, glycine betaine and sugars. Dissection of the specific roles of compatible solutes in freezing tolerance is complicated by their metabolic roles in resistance to other stresses. For example, osmolyte metabolism is likely to affect cellular redox relations and sugar sensing, and these perturbations will have secondary effects (reviewed in Hare *et al.* 1998; Winter & Huber 2000), including those that impinge on freezing tolerance.

The effect of low-molecular-weight solutes on freeze-induced injury is dependent on the lipid composition of the membrane as well as on the nature of the solutes (Hincha & Crowe 1998; Hincha *et al.* 1999). Authors emphasize different biophysical aspects of low-molecular-weight solutes in combating freeze-induced dehydrative stresses. Wolfe & Bryant (1999) and Bryant *et al.* (2001) suggest that most of the specific effects of different solutes on amelioration of membrane damage can be explained by their different biophysical properties, such as their solubility, vitrification temperature, volume, hydration and partitioning. This perspective emphasizes the role of solutes lying between membranes in limiting their close approach, and thereby ameliorating the physical stresses caused by dehydration and thus lowering the temperature at which phase transitions occur. Oliver *et al.* (1998) suggest that the specific effects of different solutes are mediated by direct interaction between the solutes and the lipid molecules of the membrane. The two models are not mutually incompatible, and the relative importance of each explanation in living systems awaits resolution.

Although there are numerous studies showing a correlation between accumulation of saccharides and freezing tolerance (e.g. Ristic & Ashworth 1993; Sauter *et al.* 1996; Xin & Browse 1998; Wanner & Junttila 1999; Gilmour *et al.* 2000), there are exceptions. Some mutants that are sensitive to freezing accumulate sugars normally on exposure to low temperature, and an elevation of the soluble sugars in tobacco does not improve its freezing tolerance (Hincha *et al.* 1996). Whilst the exceptions prove that elevation of soluble sugars alone does not combat all of the stresses that freezing imposes, they do not establish that sugars play no role in freezing tolerance.

Betaines accumulate in several species in response to many stresses, including low temperature, and exogenous application of glycine betaine has been shown to enhance stress tolerance. Synthesis of glycine betaine through transgenic expression of bacterial choline oxidase has been shown to afford protection to numerous abiotic stresses, including freezing, in several plant species that do not natively synthesize this compound. The concentrations to which glycine betaine accumulates in these transgenics are too low for its role as an osmolyte to account for these effects, and it is possible that improvement of at least some aspects of stress tolerance are due to secondary effects on stress-regulated gene expression. However, glycine betaine may also have direct effects relevant to freezing stress: for example, in transgenic *Synechococcus* the liquid-crystalline to gel phase transition temperature of the plasma membrane was depressed without affecting lipid desaturation, and *in vitro* studies have shown that glycine betaine can stabilize protein structure (reviewed in Sakamoto & Murata 2001).

Accumulation of proline is a common feature of cold acclimation, and studies in wheat have indicated that improved frost tolerance is associated with proline overproduction (Dorffling *et al.* 1997). The *esk1* constitutively freeze-tolerant *Arabidopsis* mutant has constitutively high

levels of proline, and further enhancement of freezing tolerance following cold acclimation is associated with 30-fold higher levels of proline than in the wild-type (Xin & Browse 1998). *In vitro* evidence for a direct role of proline in protection against freeze-induced lesions includes specific effects on the plasma membrane during osmotically induced contraction and reduced loss of osmotic responsiveness in proline compared with sorbitol (reviewed in Steponkus 1984), as well as reduced solute loading into thylakoids (Popova *et al.* 1998). In common with almost all compatible solutes, proline has metabolic and signalling roles as well as direct effects on freeze-induced stress: unravelling the various secondary effects from the direct protective effects is a challenging problem.

Compatible solutes have either to be manufactured in the cellular compartment where they exert their activity, or require transporters to allow them passage across the relevant membranes. Interestingly, a number of transporters of compatible solutes, some of which will carry a broad range of solutes, have been demonstrated to be cold induced (Baldi *et al.* 1999; Schwacke *et al.* 1999; Igarashi *et al.* 2000).

(c) *Cryoprotective proteins*

Many of the most abundant products of cold-induced genes are boiling-stable polypeptides related to the LEA proteins (reviewed in Hughes & Dunn 1996; Thomashow 1999). These proteins can accumulate up to 0.9% of total soluble proteins in winter wheat, after 21 days of cold acclimation (Houde *et al.* 1995). They have been found associated with many organelles, including the nucleus, endoplasmic reticulum (Ukaji *et al.* 2001) and mitochondria (Borovskii *et al.* 2000). The consistent association of these proteins with dehydrative stress across a range of higher and lower plants, as well as cyanobacteria (Ingram & Bartels 1996), has led to speculation that they could have a detergent-like activity, coating hydrophobic surfaces and preventing the coagulation of macromolecules. Dehydrins have been shown to be cryoprotective to the freeze-labile enzyme lactate dehydrogenase (Wisniewski *et al.* 1999) and it has been suggested that LEA proteins could function as chaperones in dehydrated cells. In fact, traditional chaperonin expression and activity are affected by low temperature (Anderson *et al.* 1993; Li *et al.* 1999; Ukaji *et al.* 1999; Mendoza *et al.* 2000) as might be expected in the environment of a freeze-dehydrated cell where proteins are likely to be denatured.

The function of many abundant cold-induced gene products remains speculative, but one, *Arabidopsis COR15*, has been shown to affect freeze-induced lamellar to HexII transition (Artus *et al.* 1996; Webb *et al.* 1996; Steponkus *et al.* 1998). *COR15* is an α-helical amphipathic polypeptide localized in the chloroplast stroma. It is thought that this polypeptide decreases the propensity for freeze-induced formation of the HexII phase at the inner membrane of the chloroplast, probably by altering its intrinsic curvature (Steponkus *et al.* 1998). Since the chloroplast inner membrane is composed of lipids that have the greatest propensity to form the HexII phase, prevention of HexII formation at this point prevents its propagation into closely apposed membranes. Although expression of *COR15* depresses the temperature at which HexII transition occurs, it does not eliminate it, suggesting that other cold-induced adaptations such as alterations in lipid composition, accumulation of compatible solutes or products of other cold-induced genes contribute to stabilization of the lamellar phase of chloroplast membranes.

In another approach to identifying proteins that might have cryoprotective activity, extracts from cold-acclimated plants that are known to be freeze-tolerant have been assayed for protection of membrane-bound organelles from non-acclimated plants. Newton & Duman (2000) purified an osmotin-like protein from cold-acclimated *Solanum dulcamara* that showed a cryoprotective effect towards non-acclimated kale protoplasts. Hincha *et al.* (1993, 1997*a*,*b*) showed that galactose-specific lectins from mistletoe protected thylakoids against freeze–thaw damage and that they reduced solute leakage and lipid fluidity. The same author has also identified a member of the non-specific lipid-transfer protein gene family from cabbage as a protein that is cryoprotective to thylakoids (Hincha *et al.* 2001) as well as β-1,3-glucanase from spinach (Hincha *et al.* 1997*c*). A common factor of all these proteins is that they are usually located in the apoplast and are therefore more likely to be exposed to the outer surface of the plasma membrane, a bilayer whose lipid constituents are very different from those of thylakoids. The effect of proteins on freezing damage to membranes is known to be affected by the nature of the lipid species of which the membrane is composed (Uemura *et al.* 1996; Tomczak *et al.* 2000, 2001). The *in vitro* protection of thylakoids from freezing damage by cold-induced apoplastic proteins is interesting, but it is not clear how they could perform this function *in vivo*, and whether this protection could also be afforded to the membranes to which they have access.

(d) *Ice in the apoplast*

Crystallization of water in the apoplast has been demonstrated repeatedly and can be detected as an exotherm occurring at temperatures above −6 °C that is not lethal in freeze-tolerant tissues. The deposition of ice can also be demonstrated microscopically. Upon nucleation, ice propagates rapidly through the apoplast, and this can be visualized in real time using infrared video thermography (Wisniewski *et al.* 1997; Pearce & Fuller 2001). In addition to its dehydrative effect, the crystallization of water in the apoplast imposes a mechanical stress both on the cells that it surrounds and on the fabric of the extracellular matrix.

The properties of the extracellular matrix affect both ice propagation and water loss, and this can be important for the survival of cell types that use supercooling as a means to survive sub-zero temperatures. An example is the xylem ray parenchyma cells of many tree species. In these species ice crystallizes in the apoplast of the outer layers of the bark, with large extracellular ice crystals observable in the cortex (Ashworth *et al.* 1988) but the xylem ray parenchyma cells supercool and, at temperatures above −40 °C, do not dehydrate in response to the extracellular ice elsewhere in the plant (Malone & Ashworth 1991; Ristic & Ashworth 1995). In order for these cells to supercool in this environment they must have no intrinsic ice nucleators and must have an extracellular barrier to ice propagation into the cell and water movement out of the cell. Wisniewski *et al.* (1987, 1991*a*,*b*; Wisniewski & Davis 1989) showed that the cell wall of xylem ray parenchyma

cells exhibited low porosity, which was partly attributable to the pectin components of the pit membrane structures together with the amorphous layer that surrounds this cell type. It was suggested that these presented a barrier to ice propagation into the cells and a barrier to water movement out.

Deposition of ice in other plant tissues is also regulated. In species such as forsythia and peach, ice crystallizes in the bud scales, sepals, peduncle and lower portions of overwintering dormant flower buds, but not within the developing petals, stamens or pistil. De-acclimation in the spring alters this pattern of ice deposition and ice can be detected in the floral organs. In acclimated buds, although ice crystallizes in the subtending twig, it does not propagate into the floral organs whereas it does following de-acclimation. This change in ice deposition is associated with the development of xylem continuity with the floral organs (Ashworth *et al.* 1989, 1992; Ashworth 1990). It may be related to the properties of the cell wall separating the bud from the subtending twig (Jones *et al.* 2000).

The freezing and propagation of ice in the apoplast will be affected both by the gross architecture of the tissue in question and by the nanostructure of the extracellular matrix. Ashworth & Abeles (1984) showed that pore diameters of less than 100 nm restricted the propagation of ice from one chamber to another and that water held within pores of 7.5 nm remained liquid at $-10\,^{\circ}C$. Although there are large apoplastic spaces present in some plant tissues, such as xylem vessels, the intercellular spaces of leaf tissue and the aerenchyma of roots and stems, plants also contain dense tissues where cells are closely packed and cell walls are continuous with each other. Microcapillaries within the cell wall structure are *ca*. 4 nm (Preston 1974) and are likely to significantly depress the freezing and melting point of water contained within them, and present a barrier to the propagation of ice from one large space to another. The pectin matrix is thought to control cell wall pore size (Carpita *et al.* 1979) and pore size has been shown to reduce on cold acclimation (Rajeshekar & Burke 1996).

Ice deposition in the apoplast is also probably influenced by proteins located in this compartment. In common with insects (Duman 2001), fishes (Fletcher *et al.* 2001) and other organisms that are adapted to survive in very low-temperature environments, a number of plant species have been shown to express antifreeze proteins in response to low temperature (Urrutia *et al.* 1992; Duman & Olsen 1993; Griffith *et al.* 1997; Meyer *et al.* 1999; Smallwood *et al.* 1999; Wei *et al.* 1999; Doucet *et al.* 2000; Raymond & Fritsen 2000; Sidebottom *et al.* 2000; Yeh *et al.* 2000; Fei *et al.* 2001). Antifreeze proteins interact with ice crystal surfaces, inhibiting their growth. This results in two related phenomena: (i) thermal hysteresis, the non-colligative depression of freezing temperature below the melting temperature; and (ii) inhibition of ice recrystallization, that is inhibition of the process by which large ice crystals grow at the expense of smaller ones. Although the thermal hysteresis activity of plant antifreeze proteins is low compared with that of fishes and insects, their inhibition of ice recrystallization is comparable or greater (Worrall *et al.* 1998; Smallwood *et al.* 1999). Given that antifreeze proteins are found in plant tissues where ice is allowed to crystallize in the apoplast, it has been speculated that inhibition of ice recrystallization may be the physiologically relevant aspect of their activity.

Although the expression in plants of antifreeze genes from fishes does not usually increase the freezing tolerance of species examined to date (Hightower *et al.* 1991; Kenward *et al.* 1999), some investigators have detected an effect on freezing tolerance (Wallis *et al.* 1997; Cutler *et al.* 1989). An absence of effect is not surprising since the depression of freezing temperature imparted by these proteins would be insufficient to prevent ice crystallization at relevant temperatures, and there is no reason to believe that antifreeze proteins address the particular aspect of freezing stress that kills these species. Although accumulation of antifreeze proteins has been found to correlate with winter-field survival rates in oat (Chun *et al.* 1998), it did not correlate with freezing tolerance, and it is possible that other genes that are regulated similarly to the oat antifreeze-protein genes contributed to the observed winter survival correlation.

The widespread expression of antifreeze activity amongst diverse plant species, and the universal dependence of expression on low temperature, suggests that antifreeze proteins play some role in protecting plants against sub-zero temperatures. Although some authors have emphasized the importance of antifreeze proteins in supercooling of the cytosol (Jiang *et al.* 1999), their usual location is apoplastic. Their role should therefore be considered within the context of regulation of ice growth by other components of the extracellular matrix. The growth of large ice crystals by recrystallization can mechanically damage the structure of plant tissues, especially those where cells are densely packed, and allow ice access to locations from which it is usually excluded: antifreeze proteins inhibit this growth. Equally, the depression of the freezing temperature in the small pores of the extracellular matrix may be influenced by antifreeze proteins.

6. FUTURE PROSPECTS

Rapid progress has been made over recent years in our understanding of the endogenous factors regulating the process of cold acclimation. The application of new technologies, such as microarray analysis of the transcriptome (Seki *et al.* 2001) and proteome, will probably accelerate progress in this area. This may advance our understanding of what complement of genes enhances freezing tolerance most effectively, and help to differentiate the specific roles of individual proteins, or their products, in combating sub-zero temperatures. In addition to the biotechnological potential for cultivation of agronomically important crops at increased latitude, altitude and season, the fundamental research into frost tolerance mechanisms may identify molecules that have applications in industrial settings (Dunwell 1998, 1999; Feeney & Yeh 1998; Fletcher *et al.* 1999).

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Discussion

C. Gerday (*Department of Biochemistry*, *University of Liège*, *Liège*, *Belgium*). When you are talking about cryoprotectant proteins, are you also talking about ice nucleating proteins?

M. Smallwood. What we meant by cryoprotectant proteins were proteins which you could show had an effect on the freeze stability of enzymes or on their behaviour on membranes. In a wider sense, you could regard nucleating proteins as cryoprotectant because they are ensuring that ice crystallizes in an appropriate place in an organism.

C. Gerday. And are they similar to the insect ice-nucleating proteins?

M. Smallwood. There has been very little work done on plant nucleating proteins. We know they involve protein and carbohydrate components but that is about all.

M. A. Marahiel (*Department of Chemistry*, *Philipps-Universität Marburg*, *Marburg*, *Germany*). For those proteins which are moving from the cytosol to the nucleus, do you have any evidence for post-translational modification, because as you told us these proteins are present all the time?

D. J. Bowles. Yes, there is no evidence, as yet, in Zhu's work on hos1 for a post-translational modification. Clearly, this could involve phosphorylation or dephosphorylation and I am sure that is what he will be investigating next. There is a nuclear targeting signal in the hos1 sequence and it may simply be an uncovering of that sequence, or alternatively the possibility of an interaction with a second protein, that would then carry hos1 into the nucleus. As yet, we do not know.

P. L. Davies (*Department of Biochemistry*, *Queen's University*, *Ontario*, *Canada*). Do you have any information about the three-dimensional structure of the carrot antifreeze protein?

D. J. Bowles. I am afraid we do not.

M. Smallwood. I would just like to add that it is an awkward protein to work with because you cannot express the native protein in any of the simple expression systems.

D. J. Bowles. Because of the glycosolation and two of those three sites are occupied, it is not happy at all in *E. coli* expression systems. Then you have the problem of purifying large quantities from cold-acclimated roots, and furthermore, you have the problem of making the protein again unhappy when you degylcosylate it prior to crystallization.

Anon. I was wondering if you have tested whether the transgenic tobacco was more resistant to freezing.

M. Smallwood. Not significantly in our hands. We have also tested *Arabidopsis* and in neither case does it have really any effect on freezing tolerance, and to be honest we would not expect it to. We have some data on cold-

acclimated and non-acclimated carrot roots, which show that in the non-acclimated roots you can see damage, which we have shown fairly clearly is a result of the growth of big ice crystals actually damaging the physical structure of the root, because it is quite a dense tissue. You can let ice crystallize in a leaf, where there is lots of space and there is much less physical damage than in dense tissue like a meristem or a root. You can see enormous holes where there are ice crystals in the non-acclimated tissue, which are not present in cold-acclimated tissue. We have not yet done detailed analysis of how much water is frozen in the cold-acclimated tissue but it certainly appears frozen to the touch and naked eye. Obviously, there are going to be some changes that go on in the cell wall, such as alterations to the structure of pectins, which are going to affect ice propagation, but I think probably the anti-freeze protein is one of the things which is preventing the damage that inflicted by those big ice crystals growing.

W. Bradbeer (*Division of Life Sciences*, *King's College*, *London*, *UK*). Would you say much of the material you covered in the first part of your talk would also relate to the problems of tropical plants, say in temperate or cooler conditions?

M. Smallwood. There is some evidence that even chilling intolerant plants may use some of the same signalling mechanism as temperate plants, but I think it is very unlikely that you are going to be able to just express a transcription factor in, say, banana and make it freezing tolerant.

W. Bradbeer. I was not thinking about freezing tolerant, I was just thinking about cooler conditions, not cold conditions as in North Yorkshire.

D. J. Bowles. Would anyone else like to comment? Garry Warren?

G. Warren (*School of Biological Sciences*, *Royal Holloway*, *University of London*, *Egham*, *UK*). My understanding of the problems faced by cells in chilling versus freezing conditions is that they are quite different. On the other hand, the environmental cue in both is the same so we would expect them to have common signalling components. I think there is not really any evidence for a common mechanism to tolerance to each stress.

GLOSSARY

ABA: abscisic acid
CBF: CRT-binding factors
CDPK: calcium-dependent protein kinase
CRT: C-repeat element
DRE: drought-responsive element
DREB: DRE-binding proteins
ICE: inducer of CBF expression
LEA: late embryogenesis abundant
MAPK: mitogen-activated protein kinase
MAPKK: mitogen-activated protein kinase kinase
MAPKKK: mitogen-activated protein kinase kinase kinase
P5CS: Δ1-pyrroline-5-carboxylate synthetase
PP: protein phosphatase
RRM: RNA-recognition motif