An Overview of Cold Hardiness in Woody Plants: Seeing the Forest Through the Trees

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In response to seasonal changes in growing conditions, trees and other perennials have evolved the ability to cold acclimate as well as undergo a period of dormancy. Cold hardiness is a complex trait with several contributing factors. It is also a dynamic process that changes with time. Factors involved include both bark and wood hardiness, onset of dormancy, flower budbreak and freezing tolerance of the buds, root hardiness, the influence of roots on scion growth, the frost hardiness of current year growth versus older growth, the influence of crop load on the induction of frost tolerance, and the effect of cultural practices. The development of cold hardiness in trees can be divided into five categories: the time of induction of cold acclimation, the rate of acclimation, the degree of freezing tolerance attained, the maintenance of freezing tolerance during the winter months, and the rate of loss of freezing tolerance upon resumption of spring growth.

There is a wealth of history in the area of frost tolerance of plants that dates back to the 19th century (Burke et al., 1976; Steponkus, 1984). It is fair to say, however, that while many thousands of reports have been published on plant cold hardiness, and perhaps several hundreds of reviews, a complete and integrated understanding of the topic remains elusive. One need only conduct a historical comparison of reviews to see that while perspectives have changed, the overall landscape has remained the same (Artlip and Wisniewski, 2001; Burke et al., 1976; Chen et al., 1995; Sakai and Larcher, 1987; Steponkus, 1984; Thomashow, 1999, 2001; Wisniewski and Arora, 2000; Xin and Browse, 2000).

Early observations of plant cells freezing under a microscope (Molisch, 1897; Wiegand, 1906) led to the understanding that in acclimated plants, ice forms in extracellular spaces resulting in cytorrhysis (the simultaneous contraction of the protoplast and collapse of the cell wall due to loss of water to extracellular ice). Extending these observations, Maximov (1912) suggested that disruption of the plasma membrane was the primary cause of freezing injury. These early studies provided the foundation for research conducted by Jake Levitt and Dave Siminovitch at McGill University, under the direction of the famous botanist, G.W. Scarth. In a series of classic papers published from 1936 to 1941 (Levitt and Scarth, 1936a, 1936b; Levitt and Siminovitch, 1940; Scarth and Levitt, 1937; Siminovitch and Scarth, 1938; Siminovitch and Levitt, 1941), these scientists documented that intracellular freezing was universally lethal to plant cells but did not occur in acclimated plants. They further demonstrated that the permeability of the plasma membrane increased during acclimation and played an important role in preventing intracellular ice formation and that ice-induced cell dehydration could lead to disruption of the plasma membrane upon thawing (Burke and Nozzolillo, 2002). This latter point was perhaps not fully appreciated until the later research published by Jas Singh (a graduate student of D. Siminovitch), Agriculture Canada, and Peter Steponkus at Cornell University (see review by Steponkus, 1984). Later work on the disruption of membrane transport properties as a result of freezing injury by Jiwan Palta, University of Wisconsin, also would build on this seminal work (Palta and Li, 1978 and 1980). It is also recognized commonly that the texts by Levitt (1972, 1980) are one of the few attempts that have been made to review the literature comprehensively and to present the findings into an integrated view of stress tolerance in plants. The text by Sakai and Larcher (1987) is a more recent attempt to present a comprehensive view of frost tolerance.

D. Siminovitch and his graduate student, Keith Pomeroy also were among the first to document that the protoplasm underwent distinct biochemical changes during cold acclimation that presumably played a direct role in conferring stress tolerance (Pomeroy and Siminovitch, 1971). Research by the Russian scientist I. Tumanov (as reviewed in Sakai and Larcher, 1987) also recognized the importance of biochemical changes during cold acclimation. Truly, the research conducted by these pioneers in the first half of the century formed the conceptual basis of much of what was to follow.

Regarding cold hardiness research, it is also important to recognize the important contribution that was made by the Plant Cold Hardiness Laboratory at the University of Minnesota, St. Paul, beginning in the 1960s, under the leadership of Conrad J. Weiser. Research (as reviewed in this article) conducted by scientists and graduate students at this laboratory dominated the literature for over 25 years (1960–85). A greater understanding of deep supercooling, the biophysics of water at low temperatures, dormancy, the role of sugars in cold hardiness, and more, all grew from the activities of this laboratory. People such as Paul Li, Leslie Fuchigami, Harvey Quamme, Lawrence Gusta, Milon George, Michael Burke, Jiwan Palta, John Carter, Cecil Stushnoff, Margaret Smithburg, Tim Hall, Stan Howell, Phil Graham, Robert McLeester, Bob van Huystee, and others were associated with the Plant Cold Hardiness Laboratory and would go on to staff many of the horticulture departments in the U.S. and Canada. The seminal paper by Weiser (1970) often is cited for its recognition that changes in proteins during cold acclimation also implied changes in gene expression. “Bud” Weiser later went on to chair the Department of Horticulture at Oregon State University. In the latter part of the 20th century, characterizing the genetic regulation of cold hardiness has dominated the literature (Thomashow, 1999; Xin and Browse, 2000).

Strategies allowing plants to survive freezing temperatures have been organized into two categories (Levitt 1980): 1) freezing tolerance and 2) freezing avoidance. Mechanisms representing both categories are common in woody plants (Burke and Stushnoff 1979). In comparison to herbaceous plants, woody species are extremely freezing tolerant. Many species native to boreal forests tolerate -40°C and some can even tolerate -196°C in midwinter. In contrast, very few herbaceous plants tolerate temperatures less than -25°C or prolonged exposure to -15°C for a period of several weeks, whereas certain woody species can tolerate -40°C for months.

In addition to a historical review, the present contribution will cover the main factors involved in woody plant cold hardiness, although reference will also be made to herbaceous plants where applicable. This is especially true for recent advances in the molecular biology of cold acclimation where research with *Arabidopsis* and other herbaceous crop species has dominated the literature.

ICE NUCLEATION AND PROPAGATION

Ice nucleation must first occur for ice to form on or within a plant. While this may seem like a simple matter, in fact it is quite complex, and a great deal of research has been conducted on the role of intrinsic and extrinsic ice nucleators in inducing plants to freeze. The ability to supercool tissues several degrees below zero is a form of freezing avoidance. An excellent and very comprehensive review of this topic has been presented by Lee et al. (1995). The role of ice nucleators in inducing ice formation in plants is important because if methods can be developed for regulating ice nucleation, significant advances could

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be made in limiting frost injury to both freezing-sensitive plants and flowers of many fruit crops.

Beginning in the late 1970s, a considerable amount of research focused on the role of ice nucleating agents in inducing plants to freeze at warm subzero temperatures. Much of this research was conducted by Steve Lindow at the Univ. California, Berkeley, and Edward Ashworth, USDA-ARS (now at Purdue University), and is reported in their seminal papers (see reviews by: Lindow, 1995; and Ashworth and Kieft, 1995). The working hypothesis was that by controlling extrinsic nucleation events, plants could supercool well below 0 °C and thus avoid freezing (Lindow, 1995). It was felt that such a strategy could provide a significant level of frost protection to frost sensitive plants or plant parts. In fact, the first environmental release of a genetically modified organism for use in agriculture was a strain of ice-nucleating active (INA) bacteria (*Pseudomonas syringae*) in which a large portion of the gene coding for the nucleation protein was deleted, thus rendering a nonfunctional protein and a phenotype in which ice nucleation activity was absent. This approach was perceived as very controversial by the general public and issues regarding its use filled the headlines of many major newspapers in the early 1980s. In fact, due to regulatory and legal battles, 5 years elapsed between the filing of the permit request and the actual field test (see Lindow, 1995). Society is still wrestling with the application of genetic engineering to production agriculture.

While the majority of reports dealt with the role of ice-nucleating-active (INA) bacteria, related research focused on the role of other extrinsic nucleating agents and whether or not plants could actually supercool to temperatures several degrees below 0 °C (Ashworth, 1992). Intrinsic nucleators that are active at warm subzero temperatures have been documented, especially in woody plants (Ashworth and Kieft, 1995). The presence of these internal nucleators limit the ability of a plant to significantly supercool below 0 °C even in the absence of extrinsic nucleating agents. The identification of a wide range of both extrinsic and intrinsic ice nucleating agents has made complex the practical application of blocking extrinsic ice nucleation.

Recently, infrared video thermography has been used to directly observe ice nucleation (i.e., initial ice formation) and propagation in plants (Ball et al., 2002; Carter et al., 2001, 1999; Fuller and Wisniewski, 1998; Pearce and Fuller, 2001; Wisniewski et al., 1997; Wisniewski and Fuller, 1999; Workmaster et al., 1999). This technology offers distinct advantages over the use of thermocouples to measure plant temperature. The temperature and spatial resolution of the devices used in these studies have enabled researchers to clearly define the initial site of ice nucleation and monitor the ice front as it spread into the surrounding tissues. Using infrared thermography, it is possible to determine the role of extrinsic and intrinsic ice nucleating agents in the freezing process, rates of ice propagation, the effect of plant structure on the freezing process, and how the specific pattern of freezing relates to visual patterns of injury.

Wisniewski et al. (1997) demonstrated that the freezing of water droplets on the surface of plants occurs as independent events, and that droplets containing INA bacteria freeze at warmer temperatures than plain water alone. The presence of these droplets could induce open flowers of peach (*Prunus persica*) and apple (*Malus domestica*) to freeze. However, intrinsic nucleators that were active at warm subzero temperatures, were also documented in these plants. In most cases, ice was initiated in the outer bark of stem tissues and then propagated into expanded primary tissues, such as flower buds or young shoots, despite the fact that these latter organs were supercooled to lower temperatures (due to radiative cooling) than the stem tissues themselves.

Detailed infrared studies of ice nucleation and propagation have been conducted in cranberry (*Vaccinium macrocarpon* Ait.) uprights and fruit (Workmaster et al., 1999) and flowering shoots of blackcurrant (Carter et al., 1999, 2001). Ball et al. (2002) provided a comprehensive report of the freezing process in attached leaves of snow gum (*Eucalyptus pauciflora* Sieb. ex Spreng.) in which they demonstrated that spatial variation in the physical properties of the leaves could affect the distribution of minimum leaf temperatures and hence, the distribution and extent of injury.

Collectively, the above-mentioned studies indicate that barriers to ice nucleation and propagation exist in plants and that plant structure can play an important role in frost resistance (Wisniewski et al., 2002b).

Furthermore, they indicate that the creation and/or enhancement of barriers could be used to improve frost protection. Such an approach could involve both breeding to enhance specific anatomical or structural traits, or the physical application of materials that provide a barrier to ice nucleation. The latter approach recently has been explored in herbaceous plants (Wisniewski et al., 2002a) when a hydrophobic particle film was applied to tomato (*Lycopersicon esculentum* L.) plants before the application of water containing ice-nucleation-active bacteria. In an environmental chamber, noncoated plants froze at approximately -2.5 °C, while coated plants supercooled to temperatures as low as -6.0 °C. The use of this technology to protect flowering shoots of fruit trees or other woody plants, however, may be limited due to the presence of intrinsic nucleating agents. Infrared thermography has also been shown to be useful in studying freezing events in conifers (Sutinen et al., 2001).

DEEP SUPERCOOLING AND GLASS FORMATION

Deep supercooling, another avoidance mechanism, can be defined as the ability of a population of cells or entire organs to retain cellular water in a liquid phase at low, subfreezing temperatures by remaining free from internal, heterogeneous ice nuclei and isolated from the nucleating effect of extracellular ice (Burke and Stushnoff, 1979). Deep supercooling of flower buds and xylem tissues has been reviewed by several authors (Ashworth, 1992, 1996; Fujikawa and Kuroda, 2000; Quamme, 1995; Quamme et al., 1995; Wisniewski, 1995; Wisniewski and Arora, 1993, 2000; Wisniewski and Fuller, 1999). Supercooling of floral buds stands in contrast to extraorgan freezing where water migrates to sites of extracellular ice away from the bud primordia (Sakai and Larcher, 1987) and equilibrium freezing in bark and xylem tissues where cells lose water to ice crystals present in the surrounding extracellular space (Chen et al., 1995). In both of the latter cases, the amount of water that is lost from a cell is directly dependent on the vapor pressure (and hence temperature) of the ice in the surrounding tissue. The lower the temperature, the more cellular water that is displaced. As a result, the concentration of solutes present in the cell increases and the freezing point decreases. In the present review, only deep supercooling of xylem tissues will be discussed and the reader is referred to other reviews for details on deep supercooling of floral buds vs. extraorgan freezing.

Of the many aspects of plant cold hardiness, deep supercooling is perhaps the most enigmatic (Wisniewski and Arora, 2000). The ability of some plants to maintain symplastic water in an unfrozen condition and without movement of the water into the apoplast is a remarkable adaptation that has not failed to impress both biophysicists and plant physiologists. Wiegand (1906) observed, in trees, deep supercooling down to -26 °C, and Scarth and Levitt (1937) postulated that in order for supercooling to occur in plants, the liquid mass had to be broken up into droplets or capillary columns and the resulting units or cells needed to be separated by ice-proof barriers to prevent the spread of ice crystallization from its origin. With the advent of the use of thermocouples (Tumanov and Krasavtsev, 1962; Tumanov et al., 1969), and the development of differential thermal analysis (DTA) (Quamme et al. (1972, 1973), detailed studies on deep supercooling were initiated. The mechanism that allows small domains of water to avoid freezing, despite the presence of extracellular ice, however, remains poorly understood, partly because the properties which allow deep supercooling to occur apparently rely on the structural organization of the tissue or organ. This feature has made it very difficult to manipulate plant material in a way so as to discover the fundamental mechanism and/or properties that allow deep supercooling to occur (Wisniewski, 1995; Quamme, 1995).

Deep supercooling of xylem tissues is a common characteristic of many temperate species of woody plants. DTA revealed freezing events in xylem tissues at very low temperatures (-25 to -40 °C) the appearance of which is correlated with tissue death (George and Burke, 1977; Hong and Sucoff, 1980; Quamme et al., 1972). Because of this association, DTA has been used extensively to evaluate the degree of cold hardiness of stem tissues of many important woody species of fruit and landscape plants. It has also been suggested that the northern and elevational limits of native woody plants can be correlated with the ability to deep supercool (George et al., 1974).

In order for deep supercooling to occur a barrier must exist in xylem tissues that prevents the rapid loss of water to extracellular ice and also prevents the growth of ice crystals into living cells. In this regard, the porosity and/or permeability of the cell wall appears to play an essential role in the regulation of deep supercooling. In a series of papers, Wisniewski and Davis (1989), Wisniewski et al. (1991a, 1991b), demonstrated that the structure and composition of the pit membrane of xylem parenchyma appear to play an important role in regulating the extent of deep supercooling of xylem tissues. The pit membrane is a thin portion of the cell wall that allows for the passage of solutes and other materials, including plasmodesmatal connections, between cells. It is composed mainly of cellulose and pectic materials and unlike secondary wall material is nonlignified for at least two years of development. By chemically or enzymatically altering the composition of this layer of the cell wall, Wisniewski et al. (1991b) demonstrated that deep supercooling could be eliminated or its extent reduced. The pectic component of the pit membrane appeared to play a particularly key role in regulating its porosity, although a unique arabinogalactan-rich glycoprotein was also identified in the amorphous layer subtending the pit membrane (Wisniewski and Davis, 1995). The role of this protein is unknown. Detailed studies using cryomicroscopy conducted by Ristic and Ashworth (1993, 1994), Fujikawa and Kuroda (2000) and Kuroda et al. (2003) also support the importance of cell wall properties in determining the ability of xylem ray parenchyma to lose water when exposed to freezing temperatures.

Important to a discussion on the mechanism of deep supercooling is the rate of water movement from cells to extracellular ice and also the rate of freezing employed during freeze tests. Gusta et al (1983) collected from the field a range of woody species exposed to a 3-week period of temperatures less than -30°C . A DTA study on nonthawed samples showed a significant depression of low temperature exotherms (LTE) and in some cases a complete absence of the LTE. These results indicate that LTEs reported in many studies employing a cooling rate of $1^{\circ}\text{C}\cdot\text{h}^{-1}$ or greater may be the result of inadequate time for water in the xylem ray parenchyma cells to migrate to extracellular ice. Many of the early studies on deep supercooling employed a cooling rate of 10 to $40^{\circ}\text{C}\cdot\text{h}^{-1}$. These rates would not allow for the water potential of the cell to come into equilibrium with the vapor pressure of the ice.

Another unique response of woody plant cells to freezing temperatures is the ability to form glassed cell solutions (Chen et al., 1995; Hirsh et al., 1985). Glass formation is thought to be a natural adaptation that occurs in extremely hardy plant species that can survive cooling to -196°C in liquid nitrogen. The formation of glasses is a unique metastable condition that cannot be terminated below the glass transition temperature. Aqueous glasses are extremely viscous, brought about by a high solute (sugar) concentration at a sufficiently low temperature. In poplar (*Populus tremuloides*), glasses can form below -20°C (Hirsh et al., 1985). Although glassed solutions are extremely metastable and exhibit a high degree of supercooling and high hydrostatic tension, they are not subject to ice nucleation, solute crystallization, or water vapor cavitation so long as the solution remains below the melting temperature of the glass. Thus the cytoplasm and its contents are extremely stable and relatively unaffected by the stresses associated with low temperature and the presence of ice. The glassed state is frequently relied upon for the survival of plant tissues during cryopreservation (Steponkus et al., 1992). Slow desiccation and the formation of glasses under natural conditions may be a common response in woody plant species that experience long periods of extremely low temperatures in the range of -30 to -40°C .

PHYSIOLOGY AND GENETIC REGULATION OF COLD ACCLIMATION

Generally speaking, the major stress experienced by frozen cells is believed to be the severe dehydration and concomitant cellular changes (membrane folding, protein denaturation, increased levels of toxic solutes, etc.) resulting from cell water potential coming into equilibrium with the vapor pressure of extracellular ice. The process by which plants actively undergo changes in gene expression and biochemistry that enhance their ability to withstand low temperature and desiccation stress is referred to as cold acclimation. Cold acclimation is a two or

three stage process in woody plants (Weiser, 1970), beginning in the first stage with the onset of dormancy, triggered by short day photoperiods (Fuchigami et al., 1970; Irving and Lanphear, 1967). Overexpression of a phytochrome gene (PHY A) in hybrid aspen (*Populus tremula* $\times$ *Populus tremuloides*) reduced sensitivity to short day photoperiod, prevented leaf abscission, and inhibited cold acclimation. In addition, levels of gibberellic acid (GA) and indole-acetic acid (IAA) did not decrease in response to short day photoperiod. High levels of GA have been shown to delay or inhibit freezing tolerance (Junttila et al., 2002; Weiser, 1970). While the literature is too extensive to review here, upregulation of abscisic (ABA), increases in specific sugars and other compatible solutes, and the production of specific stress-related proteins all have been associated with cold acclimation and increases in cold tolerance (Chen et al., 1995). In particular, the research of Olavi Junttila (University of Helsinki, Finland) and his colleagues has greatly increased our understanding of the photoperiodic and hormonal regulation of cold hardiness and dormancy in trees (Junttila et al., 2002).

Considerable progress has been made in the last decade in understanding cold acclimation through the application of molecular biology techniques and the use of *Arabidopsis* as a model plant system (Browse and Xin, 2001; Guy, 1999; Kaye and Guy, 1995; Shinozaki and Yamaguchi-Shinozaki, 2000; Thomashow, 1998; Xin and Browse, 2000;). Although we are still far from having a complete understanding of how plants perceive low temperatures and transduce signals that alter gene expression and metabolism, we have greatly increased our knowledge of how plants acclimate to the cold.

While compositional changes in the lipid components of the plasma membrane, as well as its integral proteins, play an important role in the development of freezing tolerance (Lynch and Steponkus, 1987; Palta and Li, 1978, 1980; Siminovich et al., 1975; Steponkus et al., 1998), they are by no means the only mechanism contributing to cold hardiness. Early work aimed at identifying genes responsive to cold treatment involved global analysis of changes in protein abundance (Guy, 1990). The results of this approach eventually led to the application of molecular biology techniques to isolate individual cold-inducible genes, examples of which are shown in Table 1. Some of these genes are associated primarily with other aspects of cellular metabolism, such as, phosphoenolpyruvate carboxykinase (Saez-Vasquez et al., 1995) and ATPase (Orr et al., 1995). Others are involved with providing protection to the plant from other stresses, like heat-shock (Ukaji et al., 1999), reactive oxygen intermediates (Llorente et al., 2002; Seppanen et al., 2000) and pathogen attack (Yeh et al., 2000).

Cold-responsive genes likely to be involved with development of cold tolerance include those encoding enzymes responsible for the synthesis of sugars or sugar derivatives (Dejardin et al., 1999; Guy et al., 1992; Taji et al., 2002) and compatible solutes or proteins (Newton and Duman, 2000; Xing and Rajashekar, 2001). Still others appear to be associated with the dehydrative aspects of freezing stress. For example, one of the largest groups of genes associated with cold response is one encoding late embryo-abundant (LEA and LEA-like) proteins (Close, 1997). Some of these proteins have been shown to have antifreeze or cryoprotective properties, but the mechanism through which they provide such protection is unknown. These genes, and in some cases their cognate proteins, also have been shown to be seasonally expressed or cold-inducible in woody plant species (Arora et al., 1992; Arora and Wisniewski, 1994; Artlip et al., 1997; Muthalif and Rowland, 1994; Rinne et al., 1998, 1999; Sarnighausen et al., 2002; Welling et al., 1997).

Another large group of cold-responsive genes are the *COR* (cold responsive) genes [i.e., cold-induced (*KIN*), low temperature inducible (*LTI*), responsive to dehydration (*RD*)]. Although these genes are associated with the plant's response to cold, except for *COR15a* (Steponkus et al., 1998) their role in the development of cold acclimation is not clear. One reason is that overexpression of these genes does not enhance freezing tolerance at the whole plant level (Kaye et al., 1998). On the other hand, given the complexity of cold acclimation, overexpression of a single cold-responsive gene may not reflect a realistic physiological condition that can lead to whole plant cold tolerance. Reverse genetics (i.e., knockout by antisense or transposon tagging) of these genes may present a clearer picture of their role in freezing tolerance.

One of the most important questions in understanding the

Table 1. Genes associated with cold tolerance.

Specific gene or protein class	Organism	Function or identity	References
COR/KIN/LTI/CAS	<i>Arabidopsis thaliana</i>	Not known at present	Kurkela and Franck 1990; Gilmore et al., 1992; Horvath et al., 1993; Welin et al., 1994; Weretilnyk et al., 1993; Orr et al., 1992
LEA or LEA-like	<i>Brassica napus</i>	Interact with other proteins?	Kaye et al., 1998
	<i>Spinacia oleracea</i>		Monroy et al., 1993; Luo et al., 1991
	<i>Medicago spp</i>		Choi et al., 1999
	<i>Arabidopsis thaliana</i>		Tsuda et al., 2000; Ndong et al., 2002
	<i>Triticum aestivum</i>		Cai et al., 1995
	<i>Poncirus trifoliata</i>		Artlip et al., 1997
	<i>Prunus persica</i>		Ukaji et al., 2001
<i>cor tmc-ap3</i>	<i>Morus bombycis</i>	Chloroplastic amino acid selective channel protein	Levi et al., 1999
	<i>Vaccinium corymbosum</i>		Baldi et al., 1999
Glycine-rich proteins	<i>Hordeum vulgare</i>	Not known at present	Luo et al., 1992; Laberge et al., 1993
	<i>Medicago falcata</i>	Associated with signal transduction	Hong et al., 1997
Protein kinases	<i>Arabidopsis thaliana</i>		Holappa and Walker-Simmons 1995
Metabolic enzymes	<i>Triticum aestivum</i>		Saez-Vasquez et al., 1995
	<i>Brassica napus</i>	PEP carboxykinase	Lee et al., 2002
Osmotin-like gene		Enolase	Newton and Duman, 2000
	<i>Solanum dulcamara</i>	Osmotin-like protein	Dunn et al., 1991
blt4.9	<i>Hordeum vulgare</i>	Lipid transfer protein?	Dunn et al., 1990
blt14		Not known at present	Goddard et al., 1993; Capel et al., 1997
blt101, RC12a & b		Not known at present	Seppanen et al., 2000
Defense proteins	<i>Solanum commersonii</i>	Glutathione S-transferase	Llorente et al., 2002
	<i>Arabidopsis thaliana</i>	Peroxidase	

mechanism(s) of cold acclimation is the identity of the components in pathways transducing signals in response to perception of low temperature (Knight and Knight, 2001; Xiong et al., 2002b). Research in the last decade has suggested that at least one component of cold acclimation works through a mitogen-activated protein kinase (MAPK) pathway (Jonak et al., 1996; Sangwan and Dhindsa, 2002) consisting of a cascade of protein kinases that activate each other in a step-wise fashion. MAPK cascades are universal signal transduction pathways found in all organisms, and it has been shown that multiple MAPK pathways within a cell are differentially activated by a variety of external stimuli and/or stresses (Agrawal et al., 2003 and others). There is also evidence of several intracellular triggers that activate the MAPK cascade during cold acclimation. One of these involves calcium signaling (Monroy and Dhindsa, 1995), while another implicates cyclic ADP-ribose (cADP) as an internal signal (Wu et al., 1997). cADP is thought to be a primary response to ABA signaling and therefore may stimulate the ABA-dependent pathway leading to cold acclimation (Xin and Browse, 2000). Recent work in this area also suggests that calcium-stimulated activation of phospholipases C and D is an early response to cold adaptation (Ruelland et al., 2002). These enzymes create phosphorylated derivatives of inositol which, like cADP and calcium, can act as intracellular messengers. Such studies also emphasize cross-talk among various cellular pathways leading to levels of complexity that are probably necessary for flexibility in the cell's ability to respond to multiple signals simultaneously, but hinder research progress in this area. Finally, since protein kinases represent only half of the phosphate signaling mechanism, protein phosphatases, which activate or deactivate proteins by dephosphorylation, also should be associated with cold responsiveness. Indeed, Meskiene et al. (1998) have isolated a protein phosphatase 2C, which inactivates the stress-activated MAPK cascade of Jonak et al. (1996) and may serve to reset the system in preparation for the next signaling event.

MOLECULAR GENETICS OF COLD ACCLIMATION

One approach to isolating genes associated with complex traits like cold acclimation is to use molecular mapping techniques (e.g., random amplification of polymorphic DNA [RAPD] or single nucleotide polymorphism [SNP]) to saturate chromosomes with markers and facilitate the identification of traits cosegregating with them. These techniques have been successfully used in plants with extensive genetics, like maize, but have proven more difficult to use with polyploid or slow-breeding species such as woody plants (Arora et al., 2000; Bliss et al., 2002). Nevertheless, several laboratories have begun using these approaches with a variety of crop species, such as citrus (Cai et al., 1994), and

rice (Kim et al., 2000).

Another productive approach to understanding the contribution of specific genes to the development of cold hardiness is to isolate mutants in response to cold treatment. This approach highlights the benefits of using *Arabidopsis* as a model, since a number of important genes significant to the development of cold tolerance have been identified in this organism. For example, Knight et al. (1999) have characterized a mutant allele, *sfr6*, in *Arabidopsis* that suppresses cold-induction of CRT/DRE-containing genes. Another genetic locus, HOS1, encodes a RING finger protein that locates to the nucleus upon cold treatment and suppresses transcription of a number of genes, including those regulated by CRT/DRE elements (Lee et al., 2001). Xin and Browse (1998) isolated a proline-accumulating mutant called eskimo1 which conferred some ($\approx 2^\circ\text{C}$) freezing tolerance to nonacclimated plants, suggesting that the gene represses cold acclimation in its wild type state. More recently a mutation conferring freezing sensitivity, *frs1*, has been shown to represent a new ABA3 allele (*aba3-3*) and is believed to be a defect in the last stage of ABA synthesis (Llorente et al., 2000). This result supports previous observations suggesting an overlap in cold- and water deficit-sensing pathways.

REGULATION OF GENE EXPRESSION IN RESPONSE TO COLD

How are cold-responsive genes regulated? One way to control gene expression is to alter the rate of transcript initiation by influencing the activity of RNA polymerase II (RNA polII). Recognition of individual genes in response to specific stimuli or in specific organs/tissues is conferred by the interaction of various transcription factors (TFs) that bind to defined sequences in the promoter regions of the target genes and interact with RNA polII to enhance or suppress transcript initiation. Early studies of cold responsive genes, such as COR15, identified cis-acting elements which were specific for cold or dehydration responses. These DNA sequences, called C-repeat (Baker et al., 1994) or DREB (Yamaguchi-Shinozaki and Shinozaki, 1994) elements, contain a core sequence consisting of CGAC. In subsequent studies the TFs binding to these elements were identified and characterized (Thomashow, 1999). In this way a family of AP2-domain C-repeat binding factors (CBFs) was shown to be associated with response to low temperature (Gilmour et al., 1998; Liu et al., 1998), but not ABA (Medina et al., 1999). Since then, other TFs representing different classes have been shown to be associated with cold-responsive gene expression (Lee et al., 2002; Xiong et al., 2002a).

GLOBAL PATTERNS OF GENE EXPRESSION IN

RESPONSE TO COLD

As discussed above, cold acclimation is a complex process involving the expression of multiple genes. The development of new techniques has facilitated the identification of large numbers of genes responsive to a variety of stimuli, including cold treatments. For example, expression profiling using microarrays of DNA on filters hybridized with cDNAs derived from tissues after selected treatments has allowed the identification of a number of genes that are up- or down-regulated by cold (Fowler and Thomashow, 2002; Kolipara et al., 2002). In addition, two-dimensional gel electrophoresis of proteins is once again being used to profile the proteome, which is all the polypeptides expressed in a given tissue under a given set of stimuli, and has recently been applied to the response of flax seedlings to cold shock (Tafforeau et al., 2002).

GENETIC ENGINEERING APPROACHES

In an effort to understand the contribution of various individual genes to the development of cold hardiness, a number of researchers have analyzed overexpression of particular genes to determine the effects on cold responsiveness or cold tolerance. For example, ectopic expression of the seed-specific transcription activator, ABI3, enhances freezing tolerance in vegetative tissues of *Arabidopsis* (Tamminen et al., 2001). In addition, overexpression of CBFs upregulates a suite of genes and has been shown to enhance freezing tolerance (Gilmour et al., 2000; Jaglo-Ottosen et al., 1998; Kasuga et al., 1999). An interesting application of this strategy is the ectopic expression of an invertase inhibitor to prevent cold-induced sweetening in potato tubers during storage (Greiner et al., 1999). Antisense inhibition has also been successfully applied to alter cold acclimation. In *Arabidopsis* antisense inhibition of protein phosphatase 2C accelerated cold acclimation (Tahtiharju and Palva, 2001). Finally, expression of foreign cryoprotective proteins has also been used successfully to protect against cold extremes (Hightower et al., 1991; Huang et al., 2002), although the protection provided has been modest in most cases.

Application of molecular techniques has been key in our initial understanding of the events involved with the development of cold acclimation in plants. Identification of the proteins involved in signaling cold perception will help researchers understand their contribution to cold acclimation, and unraveling their interactions with other pathways will continue to represent a real challenge. Since transcription factors themselves are prone to activation/deactivation by virtue of their interaction with activators and repressors, identifying proteins that interact with various cold-responsive TFs may facilitate our ability to separate out cold-specific responses from responses occurring through other pathways, like drought, ABA, and light. This information could enable us to genetically engineer enhanced freezing tolerance without affecting other important defense or developmental processes.

SUMMARY

The history of plant cold acclimation research is long and extensive. In a review on the role of the plasma membrane in freezing injury and cold acclimation, Steponkus (1984) noted that Roberts and Miska (1980) provided a bibliography on plant cold hardiness that contained 2,900 references published between 1965 to 1975, with an addendum of an additional 743 titles for completeness. Since that time it seems that the number of scientific publications has increased at an exponential rate, as have the technologies for conducting plant research. In regards to the cold acclimation of woody plants, one can ask whether or not we are any closer to understanding cold hardiness in a comprehensive manner. Can we see the forest through the trees?

In the last ten years or so, ~75% of the research done on plant cold hardiness has been conducted on a weed species, *Arabidopsis thaliana*, that can only cold acclimate by 5 to 8 °C. This research may pertain only to cold tolerance that allows for brief exposures to episodes of frost experienced by frost-sensitive species. It is highly probable that the mechanisms that allow trees to withstand extremely low subzero temperatures for long periods of time may be completely different. Even with the advent of molecular biology, genomics, proteomics,

and metabolomics, considerable emphasis will need to be placed on understanding individual gene function and biochemistry. Great progress has been made in understanding cold acclimation in woody plants and plants in general. As with every new generation, the goal appears to be attainable and just out of reach and as with every past generation, one can only stand in awe of what the future promises.

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