

Update on Adaptation to Physical Stress

Role of Cold-Responsive Genes in Plant Freezing Tolerance¹

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Plants vary greatly in their ability to survive freezing temperatures. At one extreme are plants from tropical regions that have virtually no capacity to survive even the slightest freeze. In contrast, herbaceous plants from temperate regions generally survive freezing temperatures ranging from -5°C to -30°C , depending on the species, whereas perennials in the boreal forests routinely survive winter temperatures below -30°C . Significantly, the maximum freezing tolerance of plants is not “constitutive” but is induced in response to low, nonfreezing temperatures (below approximately 10°C), a phenomenon known as “cold acclimation.” Wheat plants grown at normal warm temperatures, for instance, are killed by freezing at about -5°C , but after cold acclimation, they can survive freezing temperatures as low as -20°C .

What accounts for the differences in freezing tolerance among plant species and the increase in freezing tolerance that occurs with cold acclimation? Determining the answers to these questions is not only of basic scientific interest, but also has potential practical applications. Freezing temperatures periodically cause significant losses in plant productivity and limit the geographical locations where crop and horticultural plant species can be grown. Despite considerable effort, traditional breeding approaches have resulted in only modest improvements of freezing tolerance. For example, the freezing tolerance of the most hardy wheat varieties today is essentially the same as that of varieties developed in the early part of this century (Thomashow, 1990). Knowledge of the molecular basis of freezing tolerance and the cold acclimation process could potentially lead to the development of new strategies to improve plant freezing tolerance and result in increased plant productivity and expanded areas of agricultural production.

In 1985, Guy et al. established that changes in gene expression occur during cold acclimation. Since then, a major goal in cold acclimation research has been to identify cold-responsive genes and to determine whether they have roles in freezing tolerance. The thought has been that many cold-responsive genes probably mediate biochemical and

physiological changes required for growth and development at low temperature. Other genes, however, might have roles in freezing tolerance. The primary purpose of this *Update* is to highlight recent developments indicating that cold-responsive genes do indeed contribute to freezing tolerance. To begin, however, I present background information concerning the nature of freezing injury and the general mechanisms thought to be important in freezing tolerance. Additional information about the cold-acclimation response can be found in other recent reviews (Steponkus and Webb, 1992; Thomashow, 1994; Hughes and Dunn, 1996).

CAUSES OF FREEZING INJURY

As temperatures decrease below 0°C , ice typically forms in the intercellular spaces of plant tissues. It occurs in this location, as opposed to intracellularly (which is thought to be a fatal event), in part because the intercellular fluid generally has a higher freezing point than the intracellular fluid. In addition, it may reflect the relative levels of ice-nucleating agents present inside and outside of the cells; in the absence of a heterogeneous ice-nucleating agent, water remains (in effect) in a supercooled state above -38°C , the homogeneous ice-nucleation temperature. The accumulation of ice in the intercellular spaces can potentially result in the physical disruption of cells and tissues caused in part by the formation of adhesions between the intercellular ice and the cell walls and membranes (Levitt, 1980). However, most of the injury results from the severe cellular dehydration that occurs with freezing (Levitt, 1980; Steponkus and Webb, 1992). At a given subzero temperature, the chemical potential of ice is less than that of liquid water. Thus, when ice forms intercellularly, there is a decrease in water potential outside the cell. Consequently, there is movement of unfrozen water down the chemical potential gradient from inside the cell to the intercellular spaces. The net amount of water movement required to bring the system into chemical equilibrium depends on both the initial solute concentration of the intracellular fluid and the subzero temperature, which directly determines the chemical potential of the ice. At -10°C , more than 90% of the osmotically active

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Abbreviations: AFP, antifreeze protein; CBF1, CRT/DRE-binding factor 1; COR, cold-regulated; CRT/DRE, C-repeat/dehydration-responsive element; LEA, late-embryogenesis abundant.

water will generally move out of the cells to the intercellular spaces, and the osmolality (Osm) of the remaining unfrozen intracellular and intercellular water will be in excess of 5 Osm.

Freeze-induced dehydration could have a number of effects that result in cellular damage, such as the denaturation of proteins and precipitation of various molecules. However, the best documented injury occurs at the membrane level (Steponkus and Webb, 1992). Detailed analyses have demonstrated that freeze-induced dehydration can cause multiple forms of membrane lesions (Steponkus et al., 1993). At relatively high freezing temperatures, between about -2°C and -4°C , the predominant injury in nonacclimated plants appears to be "expansion-induced lysis," which is caused by the osmotic contraction and expansion cycle that occurs with freezing and thawing. At lower temperatures, between about -4°C and -10°C , the predominant form of injury in nonacclimated plants is freeze-induced lamellar-to-hexagonal II phase transitions, an interbilayer event involving the fusion of cellular membranes. At temperatures below -10°C , with the consequent lower water potentials and more severe dehydration, other forms of membrane damage can occur, including "fracture-jump lesions."

FREEZING-TOLERANCE MECHANISMS

The mechanisms responsible for freezing tolerance are not well understood. It is not yet possible, for instance, to engineer a freezing-tolerant tomato from first principles, let alone increase the freezing tolerance of winter wheat (about -20°C) to that of winter rye (about -30°C). Mechanisms that could potentially contribute to freezing tolerance would include helping to prevent or reverse freeze-induced denaturation of proteins, preventing molecules from precipitating, and lessening direct physical damage caused by the accumulation of intercellular ice. What is certain, however, is that cold acclimation involves the stabilization of membranes against freeze-induced damage. Indeed, whereas plasma membranes from nonacclimated plants suffer expansion-induced lysis and formation of hexagonal II phase lipids upon freezing, membranes from cold-acclimated plants do not (Steponkus and Webb, 1992). The stabilization of membranes against freeze-induced injury appears to involve multiple mechanisms. Steponkus et al. (1993) have provided compelling evidence that the increase in membrane-freezing tolerance that occurs with cold acclimation involves changes in membrane lipid composition. Alterations that can contribute to increased freezing tolerance include increased levels of fatty acid desaturation in membrane phospholipids and changes in levels and types of membrane sterols and cerebrosides. In addition, the accumulation of Suc and other simple sugars that typically occurs with cold acclimation seems likely to contribute to the stabilization of membranes, since these molecules can protect membranes against freeze-induced damage *in vitro* (Anchordoguy et al., 1987). Finally, as discussed in the following sections, there is emerging evidence that certain hydrophilic polypeptides help to stabilize membranes against freeze-induced injury.

ROLE OF COR, LEA, AND SIMILAR HYDROPHILIC POLYPEPTIDES IN FREEZING TOLERANCE

A growing number of genes have been shown to be induced during cold acclimation (Thomashow, 1994; Hughes and Dunn, 1996). Many of these encode proteins with known activities that could potentially contribute to freezing tolerance (see below). Most, however, encode either newly discovered proteins such as the Arabidopsis *COR6.6*, *COR15a*, and *COR78* polypeptides or homologs of LEA proteins such as Arabidopsis *COR47* (Table I). The polypeptides encoded by these cold-responsive genes fall into a number of groups based on amino acid sequence similarities, but all share the property of being extremely hydrophilic. In addition, many have relatively simple amino acid compositions (i.e. are composed largely of a few amino acids), have repeated amino acid sequence motifs, and remain soluble upon boiling in dilute aqueous buffer. For instance, the Arabidopsis *COR15a* gene encodes a 15-kD polypeptide that is targeted to the stromal compartment of the chloroplasts (Thomashow, 1994; S.J. Gilmore and M.F. Thomashow, unpublished data). The mature 9.4-kD polypeptide, *COR15am*, is extremely hydrophilic; it remains soluble upon boiling; it is rich in Ala, Lys, Glu, and Asp residues, which make up more than 60% of the protein; it is devoid of Pro, Met, Trp, Cys, Arg, Gln, and His residues; and it is largely made up of a 13-amino acid sequence that is repeated (imperfectly) four times. Similarly, the cold-responsive *HVA1* gene of barley (Hong et al., 1988) encodes an extremely hydrophilic 22-kD polypeptide that is rich in Ala, Thr, and Lys residues, which make up more than 50% of the protein; it is devoid of Pro, Trp, Cys, and Phe residues; and it is composed largely of an 11-amino acid sequence that is repeated (imperfectly) nine times.

The precise functions of the novel and *LEA* cold-responsive genes are not known. However, many of them are induced in seedlings in response to water deficit and ABA (Thomashow, 1994). In addition, *LEA* proteins are synthesized late in embryogenesis, just before seed desiccation (Ingram and Bartels, 1996). Based largely on these expression characteristics and the close relationship between freezing and dehydration injury, it has been speculated that these genes might contribute directly to freezing tolerance by mitigating the potentially damaging effects of dehydration associated with freezing. Recent results that support this hypothesis are summarized in the following section.

Arabidopsis *COR* Genes

Among the highly expressed cold-responsive genes of Arabidopsis are the *COR* genes, also designated *LTI* (low temperature induced), *KIN* (cold-inducible), *RD* (responsive to desiccation), and *ERD* (early dehydration-inducible; Table I; Thomashow, 1994). The *COR* genes comprise four gene families, each of which is composed of two genes that are physically linked in tandem array. The *COR78*, *COR15*, and *COR6.6* gene pairs encode newly discovered polypeptides, and the *COR47* gene pair encodes homologs of *LEA*

Table 1. Hydrophilic polypeptides encoded by *COR* genes that are induced during cold acclimation

Group	Plant	Polypeptide	Accession No. ^a	
COR6.6	Arabidopsis	COR6.6/KIN2	X55053/X62281	
		KIN1	X51474	
	<i>Brassica napus</i>	Kin1	M81224	
COR15a	Arabidopsis	COR15a	X64138	
		COR15b	L24070	
	<i>B. napus</i>	BN115	541910	
		BN26	541912	
		BN19	541911	
	<i>Brassica oleracea</i>	BoCOR	U16751	
COR78	Arabidopsis	COR78/LTI78/ RD29a	L22567/X67671/ D13044	
		LTI65/RD29b	X67670/D13044	
	<i>Spinacia oleracea</i>	CAP160	AF035535	
WCS19	<i>Triticum aestivum</i>	WCS19	481813	
	<i>Hordeum vulgare</i>	CR1	117309	
CORa	<i>Medicago sativa</i>	CORa	L03708	
		ESIPa	M74190	
CAS15a	<i>Medicago falcata</i>	GRPa	X59930	
		<i>M. sativa</i>	CAS15a	L12461
COR47 (LEA II)	Arabidopsis	COR47/RD17	X90959/ AB004872	
		LTI29/ERD10	X90958/D17714	
		LTI30/XER02	X77613/U19536	
		ERD14	D17715	
		RAB18	282880	
		<i>H. vulgare</i>	DHN5	M95810
		<i>M. falcata</i>	CAS18	L07516
			CAS17	L13415
		<i>Poncirus trifoliata</i>	COR19	2147192
			COR11	2147191
	<i>Prunus persica</i>	PCA60	U62486	
	<i>Solanum tuberosum</i>	CI7	U69633	
		<i>S. oleracea</i>	CAP85	M96259
	<i>T. aestivum</i>	WCS120	M93342	
		COR39	445603	
		WCS66	L27516	
		WCOR726	U73213	
		WCOR80	U73212	
		COR410	1169018	
	<i>Vaccinium corymbosum</i>	BBDHN1	AF030180	
HVA1 (LEA III)		<i>H. vulgare</i>	HVA1	X78205
	<i>T. aestivum</i>	WCOR615	U73217	

^a Accession numbers are for nucleic acid (those beginning with letters) and protein (those without letters) databases.

group II proteins (also known as dehydrins and LEA D11 proteins). Recent studies (see below) indicate that *COR15a* acts in concert with other *COR* genes to enhance freezing tolerance.

The *COR15a* gene of *Arabidopsis* is expressed in response to low temperature, drought, and ABA (Thomashow, 1994). As mentioned above, it encodes a 15-kD polypeptide that is targeted to the chloroplasts and pro-

cessed to a 9.4-kD polypeptide designated COR15am. To determine whether *COR15a* might have a role in freezing tolerance, Artus et al. (1996) constructed transgenic plants that constitutively express the COR15am polypeptide and compared the freezing tolerance of chloroplasts in nonacclimated transgenic and wild-type plants. The results indicated that the COR15am-containing chloroplasts in transgenic plants were 1°C to 2°C more freezing tolerant than were the chloroplasts in wild-type plants that did not contain COR15am (cold acclimation increased chloroplast-freezing tolerance about 6°C). Moreover, the effects of COR15am were not limited to the chloroplasts. Protoplasts isolated from leaves of the nonacclimated transgenic plants that constitutively produced COR15am were about 1°C more freezing tolerant at freezing temperatures between -4°C and -8°C than were those isolated from nonacclimated wild-type plants.

The results of Artus et al. (1996) indicate a role for *COR15a* in freezing tolerance. Moreover, they indicate that *COR15a* expression increases the cryostability of the plasma membrane. This conclusion comes from the fact that protoplast survival was measured by vital staining with fluorescein diacetate, a method that reports on retention of the semipermeable characteristics of the plasma membrane. However, unlike cold acclimation that increases protoplast survival over the range of -2°C to -8°C, expression of *COR15a* increased survival only over the temperature range of -4°C to -8°C (if anything, *COR15a* expression resulted in a slight decrease in protoplast survival between -2°C and -4°C). A possible explanation for this finding is that *COR15a* expression might prevent certain membrane lesions but not others. As noted earlier, the predominant form of membrane injury over the range of -2°C to -4°C appears to be expansion-induced lysis, whereas over the range of -4°C to -8°C, the predominant form of injury is freeze-induced lamellar-to-hexagonal II phase transitions (Steponkus and Webb, 1992; Steponkus et al., 1993). Thus, it is possible that constitutive expression of *COR15a* might defer the incidence of freeze-induced formation of hexagonal II phase lipids to a lower temperature, but have little or no effect on the incidence of expansion-induced lysis.

The mechanism by which *COR15a* stabilizes membranes against freeze-induced injury is not yet known. It seems unlikely that the COR15am protein has enzymatic activity, given its simple amino acid composition and primary structure. This, however, leaves open many possibilities. COR15am might interact directly with the chloroplast inner envelope and increase membrane cryostability. The location of COR15am in the chloroplast stroma is not necessarily inconsistent with protection of the plasma membrane, since formation of the hexagonal II phase is an interbilayer event that occurs largely between the plasma-membrane and the chloroplast envelope. Decreasing the propensity of the chloroplast envelope to fuse with the plasma membrane could result in less damage to the plasma membrane. Experiments to detect a direct effect of COR15am on the stabilization of membranes, however, have yielded equivocal results (Uemura et al., 1996; Webb et al., 1996). Of course, COR15am may act indirectly to stabilize mem-

branes. For example, it could potentially regulate the activity of other proteins that have roles in freezing tolerance, such as enzymes involved in sugar or lipid metabolism. Additional experiments are required to test these hypotheses.

Although constitutive expression of *COR15a* clearly enhances freezing tolerance at both the organelle (chloroplast) and cellular (protoplast) levels, the effects are modest (Artus et al., 1996). Moreover, unlike cold acclimation, *COR15a* expression alone does not result in a detectable increase in freezing survival of whole plants (Jaglo-Ottosen et al., 1998). These findings are not surprising given the results of genetic analyses indicating that freezing tolerance is a multigenic trait involving genes with additive effects (Thomashow, 1990). Indeed, multiple genes are activated by cold acclimation in Arabidopsis, including at least one member of each of the four *COR* gene pairs (Thomashow, 1994; Hughes and Dunn, 1996).

If multiple *COR* genes act in concert to increase freezing tolerance, then expression of the entire *COR* gene "regulon" would presumably increase freezing tolerance more than expressing *COR15a* alone. This hypothesis was recently tested by Jaglo-Ottosen et al. (1998). Expression of the entire battery of *COR* genes was accomplished by overexpressing the Arabidopsis transcriptional activator CBF1 (Stockinger et al., 1997). CBF1 binds to a DNA regulatory element, the CRT/DRE, which stimulates transcription in response to both low temperature and water deficit (Yamaguchi-Shinozaki and Shinozaki, 1994). The element is present in the promoters of *COR15a*, *COR78*, *COR6.6*, *COR47*, and presumably other yet-to-be-identified *COR* genes. Jaglo-Ottosen et al. (1998) found that constitutive overexpression of *CBF1* induces expression of *COR6.6*, *COR15a*, *COR47*, and *COR78* in nonacclimated Arabidopsis plants. Moreover, it results in an increase in freezing tolerance that is greater than that which occurs upon expressing *COR15a* alone. Indeed, overexpression of *CBF1* increased freezing tolerance at the whole-plant level. Taken together, the results of *COR15a* and *CBF1* overexpression indicate that the Arabidopsis CRT/DRE regulon includes freezing-tolerance genes that have roles in cold acclimation.

Spinach and Cabbage Cryoprotectins

Almost 25 years ago, Volger and Heber (1975) reported that cold-acclimated spinach and cabbage synthesize polypeptides that are highly effective in protecting isolated thylakoid membranes against freeze-thaw damage in vitro. These putative cryoprotective polypeptides were detected only in cold-acclimated plants, suggesting that they were encoded by *COR* genes. Subsequent studies by Hinch et al. (1990) indicated that the cryoprotective polypeptides act to protect membranes against freeze-induced damage by reducing membrane permeability during freezing and increasing membrane expandability during thawing. A significant limitation in all of these studies, however, was that only partially purified protein preparations were used. Thus, it was unclear whether the cryoprotective activity detected was due to a single protein or to multiple polypeptides. However, from the enrichment procedures

used, it was evident that the polypeptides, like the *COR* polypeptides, were very hydrophilic and remained soluble upon boiling.

A significant advance in the study of the spinach and cabbage cryoprotective proteins was recently made by Siegel et al. (1996), who purified a single cryoprotective protein from cold-acclimated cabbage that is effective in protecting isolated thylakoids against freeze-thaw damage in vitro. This protein, which was designated cryoprotectin, has a mass of 7 kD, remains soluble upon boiling, and appears to be encoded by a cold-inducible gene (the protein is present in cold-acclimated plants but not in nonacclimated plants). Unfortunately, there is no information concerning the amino acid sequence of cryoprotectin, and thus, it is unknown whether it is related to any of the hydrophilic polypeptides encoded by the cold-responsive genes described above. Future studies will hopefully reveal more about the nature of cryoprotectin and its mode of action in vitro and provide direct evidence about whether it has a role in protecting membranes against freezing injury in vivo.

LEA Proteins

The *HVA1* gene of barley, which encodes a LEA group III protein (also known as LEA D7 protein), is expressed during cold acclimation, in aleurone layers late in embryogenesis, and in seedlings in response to ABA and water deficit (Hong et al., 1988). Although there is no direct evidence that *HVA1* expression during cold acclimation contributes to increased freezing tolerance, there is recent evidence that the gene confers tolerance to dehydration stress. Xu et al. (1996) reported that expression of *HVA1* in transgenic rice results in increased tolerance to both water deficit and high-salinity stress. Given the relationship between dehydration tolerance and freezing tolerance, *HVA1* is a strong candidate for being a freezing-tolerance gene.

LEA D113 proteins may also have roles in freezing tolerance. Imai et al. (1996) recently demonstrated that expression of the tomato *Le25* gene in yeast increases both the freezing and high salinity tolerance of the cells. Interestingly, the protein did not impart tolerance to high concentrations of sorbitol, indicating that the *Le25* protein does not protect cells against low water potentials per se. Regardless, homologs of *Le25* seem to be good candidates for being freezing-tolerance genes. In tomato, which is a chilling-sensitive plant that does not cold acclimate, *Le25* is expressed at very low levels, if at all, in response to low temperature (Cohen et al., 1991). Whether homologs of *Le25* are expressed at high levels at low temperature in plants that cold acclimate remains to be determined.

ADDITIONAL CANDIDATE COLD-RESPONSIVE FREEZING-TOLERANCE GENES

A number of identified cold-responsive genes encode proteins with known enzymatic activities that could potentially contribute to freezing tolerance (Hughes and Dunn, 1996). For instance, the Arabidopsis *FAD8* gene (Gibson et al., 1994) and barley *blt4* genes (Hughes and Dunn, 1996), which encode a fatty acid desaturase and a putative lipid

transfer protein, respectively, are induced in response to low temperature. These genes might contribute to freezing tolerance by altering lipid composition. Cold-responsive genes encoding molecular chaperones including a spinach *hsp70* gene (Anderson et al., 1994) and a *B. napus hsp90* gene (Krishna et al., 1995) might contribute to freezing tolerance by stabilizing proteins against freeze-induced denaturation. In addition, various genes encoding signal transduction and regulatory proteins, including mitogen-activated protein kinases, calcium-dependent protein kinases, and 14-3-3 proteins, have been shown to be up-regulated in response to low temperature (Hughes and Dunn, 1996). These might contribute to freezing tolerance by controlling the expression of cold-responsive genes or by regulating the activity of proteins involved in freezing tolerance. Whether the proteins encoded by these cold-responsive genes contribute significantly to freezing tolerance remains to be determined. However, one group of proteins that accumulate during low temperature and almost certainly contribute to freezing tolerance are the recently described plant AFPs.

Plant AFPs

A recent exciting development in cold acclimation research was the finding that plants, like certain fish and insects, synthesize AFPs in response to low temperature (Urrutia et al., 1992; Griffith et al., 1997). The hallmark characteristic of these proteins is "thermal hysteresis" activity: the proteins decrease the temperature at which ice is formed but do not affect the melting point of the solution. This effect results from AFP's binding to the surface of ice nuclei and inhibiting ice crystal growth. In addition, AFPs affect the shape of the ice crystals that form when temperatures decrease below the freezing point of the solution and are potent inhibitors of ice recrystallization (they inhibit the coalescing of small ice crystals into large ice crystals).

Thermal hysteresis activity has been detected in the cell sap of cold-acclimated plants representing more than 20 species, including both dicotyledonous and monocotyledonous plants (Griffith et al., 1997). Griffith and colleagues (Antikainen and Griffith, 1997; Griffith et al., 1997) have shown that in cereals, including winter and spring rye, winter and spring wheat, winter barley, and spring oats, AFPs accumulate in the apoplastic fluid during cold acclimation (Fig. 1). AFPs do not accumulate in cold-treated maize or tobacco, which are freezing-sensitive plants that do not cold acclimate (Fig. 1). Moreover, they do not accumulate to detectable levels in the apoplastic fluids of all plants that cold acclimate. In particular, AFP activity was not detected in the apoplastic fluids of spinach and spring canola and was found at very low levels in apoplastic fluids of winter canola (*B. napus*) and kale (Fig. 1), all of which are dicotyledonous plants that can survive freezing below -14°C after cold acclimation. It is interesting that cold-acclimated kale does appear to synthesize significant levels of AFPs (Antikainen and Griffith, 1997). Thus, perhaps in kale and other dicots AFPs are primarily present intracellularly.

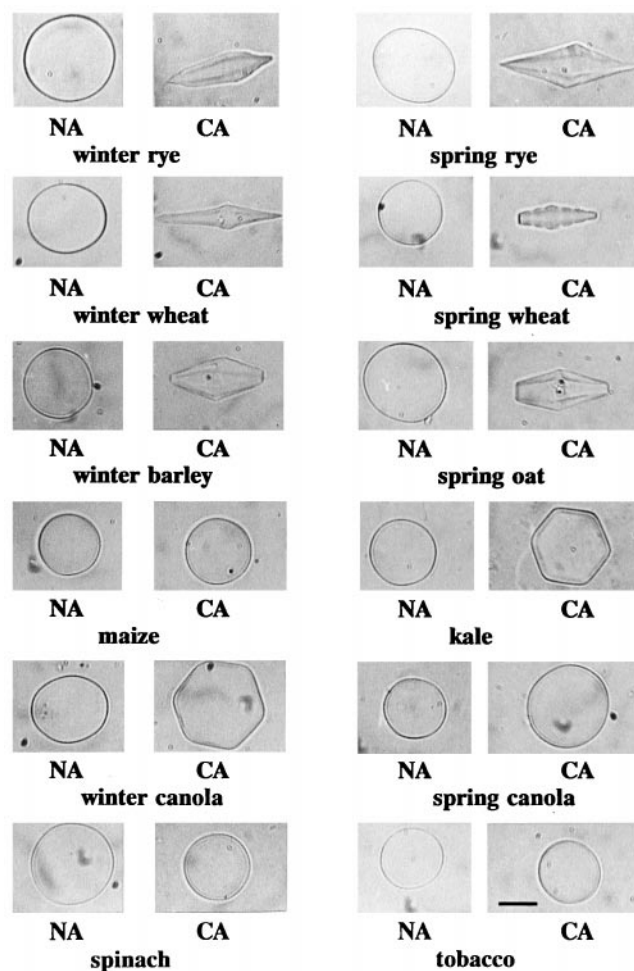


Figure 1. Antifreeze activity in apoplastic fluids from leaves of cold-acclimated (CA) and nonacclimated (NA) plants. Antifreeze activity was determined by observing the morphology of ice crystals grown in solution. All crystals were photographed at the same magnification. The bar shown on the CA tobacco sample represents $17\ \mu\text{m}$. These results are reprinted with permission from Antikainen and Griffith (1997).

Six AFPs ranging in molecular mass from 16 to 35 kD accumulate in the apoplastic fluid of winter rye during cold acclimation (Antikainen and Griffith, 1997). Surprisingly, these AFPs are related to pathogenesis-related proteins, an intriguing situation given that winter cereals have been reported to be more resistant to fungal diseases after cold acclimation (Griffith et al., 1997). N-terminal amino acid sequencing and immunoblot analyses have established that two of the AFPs are endochitinase-like proteins, two are β -1,3-glucanase-like proteins, and two are thaumatin-like proteins (Antikainen and Griffith, 1997). One of the rye AFPs has been biochemically purified and shown to have both endochitinase and antifreeze activity (the protein alters the shape of ice crystals and has a low level of thermal hysteresis activity). In contrast, a purified endochitinase from tobacco, a freezing-sensitive plant, was found to be devoid of antifreeze activity. In addition, endochitinase-, glucanase-, and thaumatin-like proteins are present in cell

extracts of nonacclimated rye plants, but no antifreeze activity can be detected. Thus, antifreeze activity is not an inherent property of these proteins. Instead, it appears that there are either specific isozymes of the rye pathogenesis-related proteins that have antifreeze activity and/or post-translational modifications convert the pathogenesis-related proteins to forms that have antifreeze activity.

It is not yet certain that the plant AFPs contribute to freezing tolerance. There are no mutants of rye or other plants, for instance, that do not produce the AFPs that can be compared with wild-type plants for freezing tolerance. However, given their known activities and accumulation during cold acclimation, it seems highly probable that they have roles in cold acclimation. The results of Marentes et al. (1993) are consistent with this notion. These investigators found that leaves from cold-acclimated rye plants that had the apoplastic proteins removed by washing were less freezing tolerant than control leaves that had not been washed free of apoplastic proteins.

How might the AFPs enhance freezing tolerance? In general, it would not appear to involve inhibition of ice formation. Indeed, the herbaceous plants in which the AFPs have been shown to exist tolerate freezing, they do not avoid it. Moreover, the thermal hysteresis activity of the plant AFPs is quite low (Griffith et al., 1997). Cell sap and apoplastic fluids from cold-acclimated plants produce only a few tenths of a degree of thermal hysteresis. A 67-kD AFP purified from cold-acclimated bittersweet nightshade (*Solanum dulcamara*) produces only about 0.3°C of thermal hysteresis at a concentration of 10 to 30 mg mL⁻¹ (Duman, 1994). In contrast, certain insects that avoid freezing synthesize AFPs that can produce as much as 7°C of thermal hysteresis at similar protein concentrations. The plant AFPs, however, are potent inhibitors of ice recrystallization, a property that occurs over a much larger temperature range than thermal hysteresis. In the case of apoplastic AFPs, it is conceivable that this property, or the effects that AFPs have on ice crystal shape, might mitigate against physical damage caused by ice and thereby enhance freezing tolerance. Intracellular AFPs, which do not come into direct contact with ice, might have fundamentally different roles. For instance, it has been demonstrated that certain AFPs from fish can inhibit the ice-nucleating activity of the ice-nucleation proteins of *Erwinia herbicola* (Parody-Morreale et al., 1988). The ability to neutralize potential ice nuclei within plant cells could help prevent intracellular ice formation, a potential problem if temperatures quickly decrease below 0°C, resulting in supercooling of both the intracellular and extracellular fluids. Testing models for how plant AFPs might contribute to freezing tolerance is clearly an important objective for the coming years.

CONCLUSIONS AND FUTURE PERSPECTIVES

There is now direct evidence that the changes in gene expression that occur with cold acclimation contribute to increased freezing tolerance. Constitutive overexpression of the Arabidopsis transcriptional activator CBF1 induces expression of CRT/DRE-regulated cold-responsive genes

and results in a marked increase in Arabidopsis freezing tolerance (Jaglo-Ottosen et al., 1998). Indeed, expression of even a single CRT/DRE-regulated gene, *COR15a*, results in a detectable increase in freezing tolerance at both the chloroplast and cellular levels (Artus et al., 1996). Thus, the fundamental issue of whether cold-responsive genes have roles in freezing tolerance now shifts to identifying which have key roles in cold acclimation and determining their modes of action. As discussed above, there are many candidate freezing-tolerant genes, including those encoding the newly discovered plant AFPs. In Arabidopsis, where it is clear that members of the CRT/DRE regulon contribute to freezing tolerance, it will be important to identify which CRT/DRE-regulated genes have roles in cold acclimation, to establish their specific modes of action, and to determine the extent to which freezing tolerance is conditioned by the CRT/DRE regulon.

A fundamental issue that remains to be clarified is whether cold acclimation involves the induction of a highly conserved set of freezing-tolerance genes. The available data do not allow for a definitive answer to this question, largely because only one cold-responsive freezing-tolerance gene has been identified, Arabidopsis *COR15a*. Homologs of *COR15a* are known to exist in *Brassica* species (Table I), but whether they exist in more distantly related plants remains to be determined. However, what is clear is that cold acclimation, from Arabidopsis to peach, is associated with the induction of cold-responsive genes that encode extremely hydrophilic polypeptides that, in many cases, have relatively simple amino acid compositions and are composed in large part of repeated amino acid sequence motifs. These polypeptides, which are either newly discovered or are homologs of highly conserved LEA proteins, are thought to protect cells against dehydration stress and, thus, potentially contribute to freezing tolerance. Evidence in favor of this hypothesis is beginning to accumulate. Expression of *COR15a* increases the freezing tolerance of Arabidopsis plants (Artus et al., 1996); expression of the barley LEA III gene *HVA1* increases the dehydration tolerance of transgenic rice plants (Xu et al., 1996); and expression of the tomato LEA D113 gene *LE25* increases both the freezing and high-salinity tolerance of yeast cells (Imai et al., 1996). Continued efforts to determine the precise modes of action of the COR, LEA, and similar hydrophilic polypeptides that accumulate with cold acclimation are critical to our understanding of freezing-tolerance mechanisms. Moreover, such information should contribute to our fundamental knowledge of how plants cope with dehydration stress in response to environmental conditions such as drought and programmed developmental events such as seed maturation.

At the beginning of this article it was noted that we do not yet have sufficient understanding of the freezing-tolerance mechanism to design more freezing-tolerant plants from first principles. However, as is hopefully evident from the research highlighted here, investigators are closing in on genes that are likely to have major roles in cold acclimation. By modifying the expression of these genes, either individually or as a group, through the use of transcriptional activators such as CBF1 or other compo-

nents of low-temperature signal transduction, we may be able to improve the freezing tolerance of agriculturally important plants. Research in the coming years should provide insight into the strengths and weaknesses of such approaches.

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