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Antifreeze proteins in overwintering plants: a tale of two activities

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Antifreeze proteins are found in a wide range of overwintering plants where they inhibit the growth and recrystallization of ice that forms in intercellular spaces. Unlike antifreeze proteins found in fish and insects, plant antifreeze proteins have multiple, hydrophilic ice-binding domains. Surprisingly, antifreeze proteins from plants are homologous to pathogenesis-related proteins and also provide protection against psychrophilic pathogens. In winter rye (*Secale cereale*), antifreeze proteins accumulate in response to cold, short day-length, dehydration and ethylene, but not pathogens. Transferring single genes encoding antifreeze proteins to freezing-sensitive plants lowered their freezing temperatures by ~1°C. Genes encoding dual-function plant antifreeze proteins are excellent models for use in evolutionary studies to determine how genes acquire new expression patterns and how proteins acquire new activities.

During winter, plants freeze at subzero temperatures (Figure 1) and form ice in intercellular spaces, xylem vessels and tracheids. Freezing injury usually arises from cellular dehydration as intracellular water is lost to the growing extracellular ice [1]. Injury can also occur when plants are frozen for prolonged periods because ice undergoes a spontaneous process known as recrystallization, in which ice crystals coalesce to minimize their surface area [2]. Although an insulating blanket of snow is protective against low temperatures, it can also cause problems by maintaining ambient temperatures near the melting point of plant tissues where ice recrystallizes quickly to form physically damaging masses of ice. Moreover, psychrophilic plant pathogens, which have optimal growth temperatures below 20°C, prosper under snow cover [3,4]. In spite of low temperatures, freeze–thaw cycles, ice recrystallization and attacks by low-temperature pathogens, many perennial and biennial plants survive winter without injury. Some of these plants secrete antifreeze proteins (AFPs) into the apoplast that inhibit the growth of extracellular ice (Figure 1 and see Table 1 in Supplementary material) and pathogenic fungi.

Interaction between antifreeze proteins and ice

Unlike most solutes that are simply pushed ahead of the ice face during freezing, AFPs bind irreversibly to the surface of ice and are incorporated into the ice crystal lattice [5]. Although it seems counterintuitive, the ice-binding domains of fish and insect AFPs are flat and relatively hydrophobic and their adsorption onto ice is a hydrophobic interaction driven by the increase in entropy gained by releasing hydration water from the ice and protein surfaces. Binding to ice is stabilized by a combination of van der Waals interactions and hydrogen bonds from hydrophilic amino acids strategically arranged to match the spacing of the ice lattice [6]. Analyses of AFPs from fish, insects and plants have shown that there is no consensus sequence or single structure for an ice-binding domain [6] and that some AFPs undergo structural changes at low temperatures [7,8]. Currently, it is not possible to identify a novel AFP from a database search; the only way to identify an AFP is by assaying its ability to interact with ice.

The binding of AFPs to ice can be monitored in several ways. First of all, the interaction is visible as a change in the morphology of ice crystals as they grow (Figure 2). Ice crystals grown in water or in a solution of substances that do not interact with ice are round and flat. By contrast, most AFPs bind to the prism faces of ice, creating hexagonally shaped crystals. A second method of assaying antifreeze activity relies on the ability of AFPs to decrease the freezing temperature of a solution by stopping the growth of ice crystals at a protein concentration that is low enough to have little effect on the melting temperature of the solution (Figure 1). The temperature range defined by the nonequilibrium freezing temperature (below which AFPs cannot stop crystal growth) and the melting temperature of the ice crystal in solution is referred to as thermal hysteresis [5]. The third technique used to study AFP–ice interactions involves monitoring the rate of recrystallization of ice by flash-freezing a solution to obtain a population of ice crystals, holding the sample isothermally just below its melting temperature, and measuring changes in crystal size over time (Figure 2). This method is the most sensitive because the recrystallization of ice is dramatically inhibited at nanomolar concentrations of AFPs. Because the rate of recrystallization can be affected by solutes other than AFPs, recrystallization experiments are generally conducted at high solute concentrations to minimize nonspecific effects [2,9].

The specific activity of AFPs, whether measured as changes in ice crystal shape, thermal hysteresis, or inhibition of ice recrystallization, varies among

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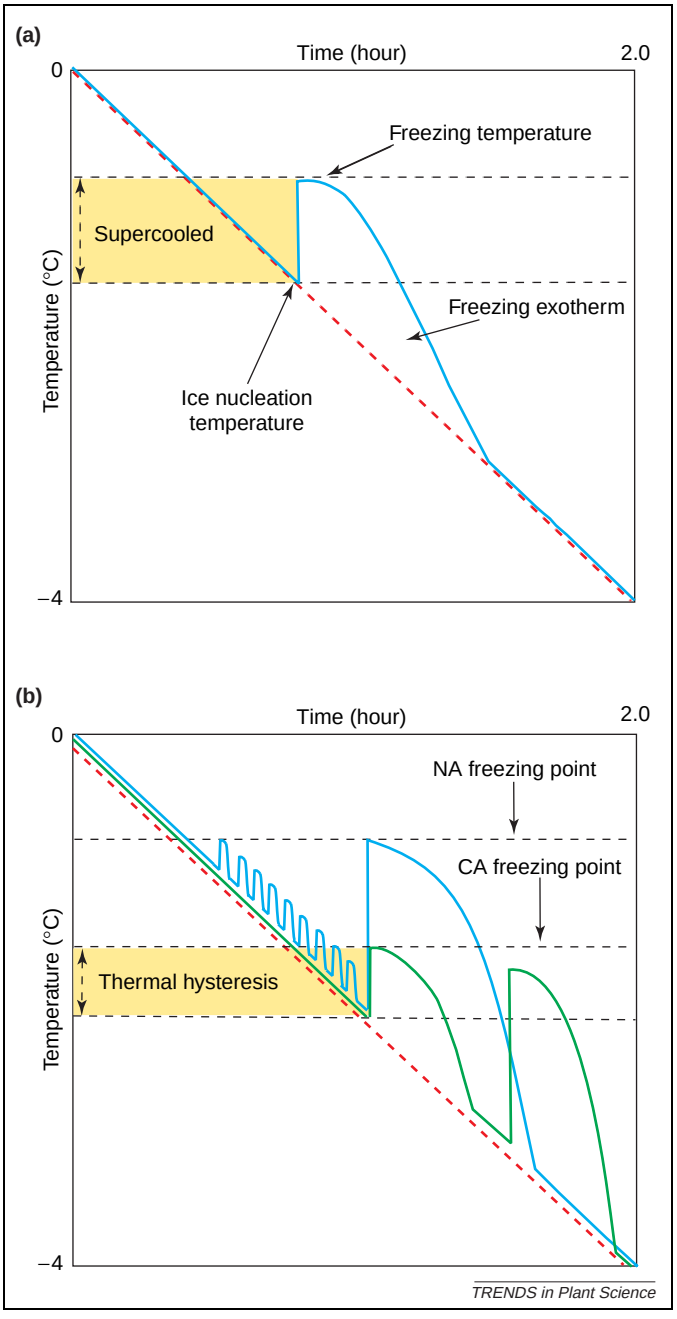

Figure 1. The freezing process. **(a)** Freezing of a nonacclimated (NA) leaf in the absence of external ice. As the ambient temperature decreases (shown by broken red line), the leaf cools until it reaches its heterogeneous ice nucleation temperature where ice nucleators organize water molecules into a nascent crystal lattice. Because the freezing of water is exothermic, the leaf temperature rises to its freezing temperature. Once the heat of fusion has dissipated, the temperature of the leaf returns to ambient. A leaf is defined as supercooled when it remains unfrozen below its freezing temperature (highlighted in yellow). **(b)** Freezing of nonacclimated (NA) and cold-acclimated (CA) winter rye (*Secale cereale*) leaves with ice present on the leaf surface. As a NA leaf cools, ice on its surface penetrates through stomates and freezes small volumes of apoplastic solution, shown by a series of small exotherms (blue line). This is followed by freezing of water in the xylem and water drawn from the symplast to the extracellular ice (large exotherm). CA leaves accumulate sugars and other solutes that lower the freezing temperature. Because CA leaves also accumulate antifreeze proteins (AFPs), ice does not grow until the leaf temperature reaches the lower temperature limit for antifreeze activity (green line). The difference between the colligative freezing point of the tissues and the depression of the freezing temperature owing to the noncolligative effects of AFPs is referred to as thermal hysteresis (highlighted in yellow). Freezing takes place in two distinctive steps in CA winter rye leaves, which could represent freezing of apoplastic water followed by water drawn from the symplast [1]. The leaves are not killed directly by freezing because the LT_{50} of NA and CA winter rye leaves is about −6°C and −20°C, respectively.

overwintering organisms and could be related to their freezing strategies. For example, AFPs produced by insect larvae that survive the winter in a supercooled state exhibit much higher levels of thermal hysteresis (3–5°C) [10] than those from freezing-tolerant plants (0.1–0.5°C) [11–13]. By contrast, AFPs from overwintering plants are particularly effective inhibitors of ice recrystallization, possibly because they have multiple ice-binding domains that can interact with more than one ice surface. For example, the AFP from perennial ryegrass (*Lolium perenne*) is predicted to fold into a β-roll with two ice-binding domains located on opposite sides of the protein [14]. Alternatively, individual AFPs with single ice-binding domains can form oligomers that consequently have multiple ice-binding domains, as is the case in winter rye (*Secale cereale*) [15]. Overwintering organisms typically produce several AFPs because each protein binds to a different plane of the ice crystal lattice and works in concert to inhibit the growth of ice [5,10,16]. Antifreeze activity can also be enhanced by interactions between AFPs and other molecules. For example, the thermal hysteresis of AFPs isolated from the larval hemolymph of the beetle *Dendroides canadensis* increased threefold in the presence of a 70-kDa activator protein and sixfold in the presence of citrate [17].

Antifreeze activity in plants

Antifreeze activity was first reported in plants in 1992 [12,13] and has since been found in many more overwintering vascular plants, including ferns, gymnosperms, and monocotyledonous and dicotyledonous angiosperms (see Supplementary material) [11,13,18,19]. Antifreeze activity is present in overwintering plants only after they have been exposed to low temperatures and only in plants that tolerate the presence of ice in their tissues. Antifreeze activity has been observed in different parts of overwintering plants, including seeds, stems, crowns, bark, branches, buds, petioles, leaf blades, flowers, berries, roots, rhizomes and tubers [11,13,18].

Plant antifreeze proteins are also pathogenesis-related proteins

AFPs have been isolated from six plants and full-length nucleotide sequences are available for genes encoding five AFPs (Table 1). The surprising result of sequencing plant AFPs, or their corresponding genes, is that most of them are homologous to pathogenesis-related (PR) proteins [20]. Normally, PR proteins are released into the apoplast in response to pathogen infection and act together to degrade fungal cell walls enzymatically and inhibit fungal enzymes. Secreted PR proteins with antifreeze activity have now been isolated from winter rye, bittersweet nightshade (*Solanum dulcamara*) and carrot, and include β-1,3-glucanases, chitinases, thaumatin-like proteins [20,21], and a polygalacturonase inhibitor protein [22,23].

Psychrophilic pathogens such as snow molds prosper under snow cover where the temperatures are nearly constant, the humidity is high [3,4,24], and it is difficult for host plants to mount a *de novo* defense at subzero temperatures. By accumulating PR proteins during cold

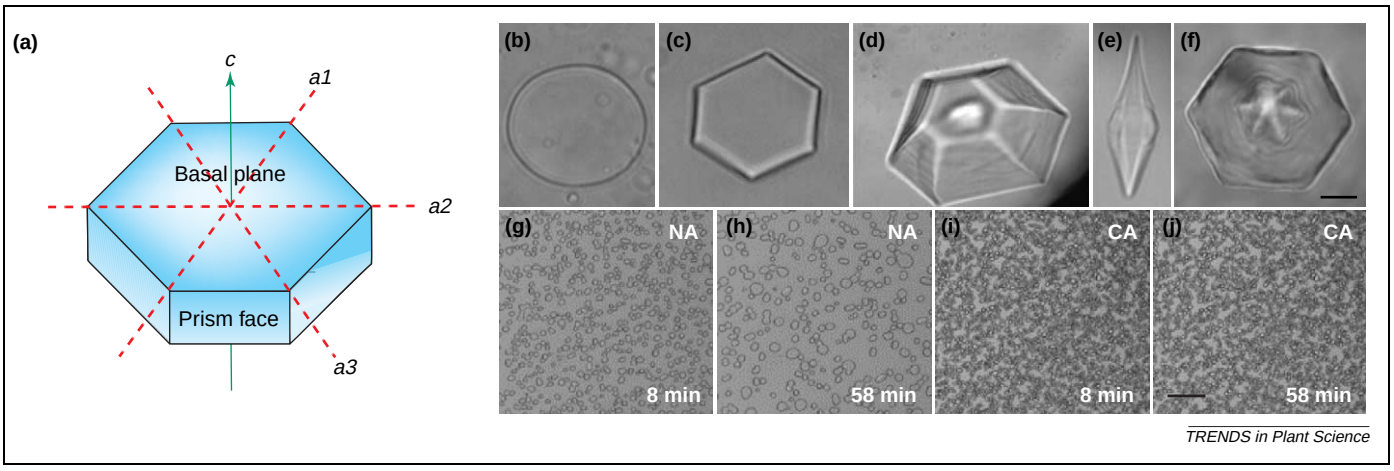

Figure 2. Assays of antifreeze activity by ice crystal morphology, thermal hysteresis and inhibition of recrystallization. **(a)** To observe changes in ice crystal morphology **(b–f)**, a solution is flash-frozen on a freezing stage of a microscope, and then warmed until only one ice crystal is present. As the temperature is lowered again, the ice crystal acquires a shape that is defined by growth along the three *a*-axes and the *c*-axis. (b) In water or in a solution of substances that do not interact with ice, ice grows as a round and flat crystal. (c) In dilute solution, most types of antifreeze proteins (AFPs) bind to the prism face of ice, creating hexagonally shaped crystals. (d) Adsorption of AFPs to the prism face inhibits the binding of additional water molecules, making it energetically favorable for water to bind to the basal plane so that the crystal grows along the *c*-axis (toward the viewer). (e) At high concentrations of AFPs, the ice crystals form bipyramids that are hexagonal in cross-section. (f) When the temperature is cooled and warmed in slow cycles, it is possible to see ridges on the surface of the ice crystal where the AFPs have bound. Scale bar = 10 μm. When the temperature stage is controlled by a freezing point osmometer, the shape of the ice crystals can be used to determine thermal hysteresis, which is the difference between the melting temperature and freezing temperature of an ice crystal in solution. After obtaining a single crystal, the stage is warmed until the crystal just disappears. This is defined as the melting temperature of the solution and is calculated from the measured osmolality of the solution. After obtaining another single crystal, the temperature is lowered slowly until the crystal suddenly grows rapidly. This is the freezing temperature. **(g–j)** Assay of the recrystallization of ice. Scale bar = 1.75 cm. (g) Total soluble extracts from nonacclimated (NA) and cold-acclimated (CA) winter rye (*Secale cereale*) leaves containing 26% sucrose were flash-frozen at −50°C to obtain a population of ice crystals and then held isothermally at −7°C to allow ice to recrystallize for 8 and 58 min. After 58 min, the extracts from NA winter rye contained fewer, larger ice crystals, but there was little effect on the number or size of ice crystals in extracts from CA leaves enriched in AFPs. Therefore, CA winter rye leaves contain inhibitors of ice recrystallization.

acclimation, overwintering grasses and cereals acquire a systemic, nonspecific, pre-emptive defense against these pathogens and exhibit greater disease resistance [3,24,25]. In winter rye, the AFPs exhibit antifungal, hydrolytic activities and ice-binding activity [20,25]. Therefore, cold-acclimated plants are more resistant to injury caused either by snow molds or by freezing (Figure 3) [25]. Genetic studies have shown that the genotypic correlation between freezing tolerance and snow mold resistance is approximately one in half-sib families of cocksfoot (*Dactylis glomerata*), indicating that the same genetic traits are involved in these two physiological processes [24].

Interestingly, winter rye plants infected by pathogens or treated with salicylic acid or abscisic acid when the temperature is warm accumulate a group of PR proteins that are similar to those that accumulate during cold acclimation, but proteins in nonacclimated plants lack antifreeze activity [25–27]. In winter rye, neither the cold-induced chitinase-AFPs nor the pathogen-induced chitinases are post-translationally modified beyond cleavage of the sequence targeting them for secretion [28], and so it is still not known what features distinguish PR proteins with and without antifreeze activity. One possibility is that AFPs and PR proteins interact with other molecules that influence their activities. For example, the hydrolytic activity of a chitinase-AFP from cold-acclimated winter rye leaves is enhanced fivefold in the presence of 20 mM Ca^{2+}, but freezing and thawing AFPs in the presence of Ca^{2+} inhibits antifreeze activity [8]. Ca^{2+} levels in the apoplast are usually low, but can be released from the cell wall upon infection to increase the hydrolytic activity of the enzymes.

At this time, there is no known homology for the partial sequence of the AFP from perennial ryegrass and no sequence information is available for AFPs from *Ammopiptanthus mongolicus*. The AFPs from carrot, bittersweet nightshade, and perennial ryegrass are all glycosylated, but only the AFP from bittersweet nightshade requires glycosylation for antifreeze activity [21,23,29,30].

The carrot AFP with sequence similarity to polygalacturonase inhibitor protein does not inhibit polygalacturonase [22,23]. About 74% of the mature carrot AFP sequence is composed of a leucine-rich repeat (LRR) region that is common in resistance (R) genes and provides rapidly evolving specificity to new pathogens [22]. It is possible that the LRR region acquired the ability to bind to ice and now the protein might function only as an AFP [22,23]. A recent model of the carrot AFP shows that it folds into a β-helix with the Leu residues stacked internally to form a hydrophobic core [31]. Unlike the fish AFPs, the ice-binding domain is strongly hydrophilic with highly conserved asparagines spaced at intervals that are complementary to a prism plane of ice. The thermal hysteresis of the carrot AFP decreased when Asn residues were replaced with Val or Glu by site-directed mutagenesis, and increased when the ice-binding face was lengthened by replacing Phe or Thr with Asn to create an additional ice-binding site [31]. It is not known how an AFP with a hydrophilic ice-binding domain interacts with ice rather than liquid water [6], but the ability of a plant AFP to make a lattice match could allow it to bind to ice nucleators as well as ice [32] and inhibit both ice nucleation and recrystallization.

Two of the AFPs that have been isolated lack secretory signals: one is an AFP from bittersweet nightshade that has a zinc-finger motif and a nuclear localization signal and shares homology with a WRKY DNA-binding protein. WRKY proteins are transcription factors that regulate the

Table 1. Antifreeze proteins that have been isolated from cold-acclimated plants

Plant	Molecular mass (kDa)[a]	Homology	GenBank[b] Accession no.	Refs
Ammopiptanthus mongolicus	50	No homology reported		[48]
Ammopiptanthus mongolicus	28	No homology reported[c]		[49]
Bittersweet nightshade (*Solanum dulcamara*)	67	WRKY protein[d,e]	AAL26842	[21]
Bittersweet nightshade	47	Chitinase-like protein[d]		[21]
Bittersweet nightshade	29	Class I chitinase-like protein[d]	AAP32201	[21]
Carrot (*Daucus carota*)	36	Polygalacturonase inhibitor protein[d]	AF055480	[9,22, 23]
Peach (*Prunus persica*)	60	Dehydrin[e]		[33]
Perennial ryegrass (*Lolium perenne*)	29	No homology reported[c]	AJ277399 (fragment)	[30,50]
Winter rye (*Secale cereale*)	35	β-1,3-endoglucanase[d]		[20]
Winter rye	32	β-1,3-endoglucanase[d]		[20]
Winter rye	35	Class I endochitinase[d]	AF280437	[20,28]
Winter rye	28	Class II endochitinase[d]	AF280438	[20,28]
Winter rye	25	Thaumatin-like protein[d]		[20]
Winter rye	16	Thaumatin-like protein[d]		[20]

[a]Molecular mass determined by SDS-PAGE.
[b]www.ncbi.nlm.nih.gov
[c]Heat-stable protein.
[d]Pathogenesis-related protein.
[e]Intracellular proteins.

production of PR proteins in plants [21]. The other intracellular AFP (PCA60) was isolated from the bark of peach trees and shows homology to dehydrins, which are proteins that accumulate in response to desiccation [33]. Because some AFPs can bind to ice nucleators as well as ice [32], the peach and bittersweet nightshade AFPs could bind to intracellular ice nucleators to prevent the initiation of intracellular ice or they could be released from cells upon injury and then interact with extracellular ice.

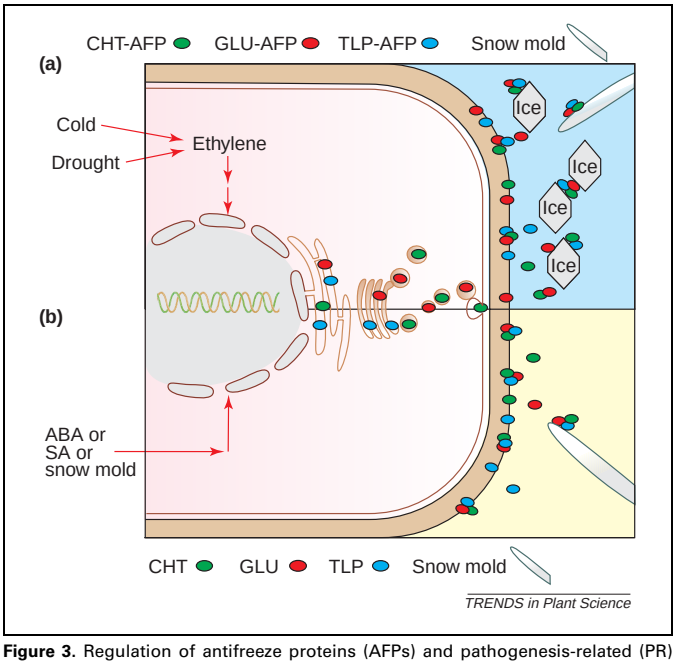

Figure 3. Regulation of antifreeze proteins (AFPs) and pathogenesis-related (PR) proteins in winter rye (*Secale cereale*). **(a)** When subjected to low temperatures or drought, winter rye plants synthesize ethylene, which induces new gene expression. Newly synthesized, dual-function PR proteins and AFPs are secreted via the endoplasmic reticulum, Golgi bodies and vesicles that merge with the plasmalemma and accumulate on the outer cell wall surface [35,40]. The proteins, including chitinase-AFPs (CHT-AFP), glucanase-AFPs (GLU-AFP) and thaumatin-like AFPs (TLP-AFP) [20], form oligomeric complexes [15] that inhibit the growth of low-temperature pathogens such as snow molds [25]. Once the temperature drops below freezing, the proteins adsorb onto the surfaces of ice and inhibit its growth and recrystallization. **(b)** When winter rye plants are treated with salicylic acid (SA), abscisic acid (ABA) or snow mold at warm temperatures, they secrete newly synthesized PR proteins into the apoplast, where they inhibit the growth of fungal pathogens [25–27]. These PR proteins are chitinases (CHT), glucanases (GLU), and thaumatin-like proteins (TLP). They are probably produced by different members of the gene families than those that produce the PR proteins and AFPs because they lack antifreeze activity and their accumulation is regulated differently.

Regulation of antifreeze proteins

To date, no plant has been reported to have constitutive antifreeze activity; rather all studies have shown that transcripts and translation products of AFP genes accumulate during cold acclimation [12,13,20,21,28]. The conditions used for cold acclimation mimic autumn when days become shorter and colder. Therefore, low temperature and daylength are important environmental cues for AFP production. For example, winter rye plants grown at low temperatures accumulate more apoplastic protein under 8-h days than under 16-h days [34].

The regulation of AFPs is complex because it also involves tissue-specific and developmental responses, as illustrated by studies of cold induction and tissue specificity of AFPs in two plants. The carrot AFP gene (*DcAFP*) was expressed within 30 min of exposure of seedlings to 4°C and reached steady-state levels within 24 h [22]. *DcAFP* transcripts were maintained at steady-state levels in leaves, stems and roots of mature carrot plants as long as they were exposed to cold and short days [9]. In winter rye leaves, glucanase-AFPs were detected immunologically within 24 h after transferring vegetative plants to 5°C and short days, but chitinase-AFPs accumulated only after 3 to 7 weeks and disappeared within 12 h when plants were returned to 20°C and long days to deacclimate [20,28]. *CHT9* and *CHT46* transcripts corresponding to chitinase-AFPs were not found in roots, but were detected throughout the leaves in parenchymal sheath, mesophyll, epidermal and phloem cells [35]. Unlike the carrot AFP, winter rye chitinase-AFPs did not accumulate directly in response to cold and/or short days, instead they accumulated only indirectly as part of the growth and development that occurs during cold

acclimation in winter cereals [28]. Moreover, only three of the six AFPs were secreted into the medium by winter rye cells grown in suspension culture at 4°C [36], demonstrating again that there is a developmental component to the regulation of AFP synthesis.

Gene families encode PR proteins: a different signalling pathway regulates each member of the family. Therefore, PR proteins with and without antifreeze activity are likely to be different members of gene families that are differentially regulated by cold and by pathogens. Many cold-regulated genes are transcriptionally responsive to abscisic acid (ABA) and drought, whereas genes encoding PR proteins are induced by several pathways, including pathogens, elicitors, jasmonate, ethylene and salicylic acid [37]. Winter rye plants produce AFPs at 20°C after drought or treatment with ethylene, but not after treatment of the plants with ABA or salicylic acid [26,27], or infection with pathogenic pink snow mold (*Microdochium nivale*) [25]. The importance of ethylene in the regulation of AFPs was confirmed by Xiao-Ming Yu *et al*. [27], who showed that winter rye plants produce ethylene within 12 h of being transferred to 5°C or within 24 h of being rewatered after drought stress, and that antifreeze activity is detectable 48 h after the start of the treatment. Moreover, treating plants with the ethylene inhibitor $AgNO_3$ blocks the development of antifreeze activity [27].

Role of antifreeze proteins in freezing *in planta*

The lower limit of freezing tolerance of a plant population is measured as LT_{50}, the lethal temperature for 50% of the individuals. In single plants, LT_{50} is often determined as the loss of 50% of the electrolytes from plant tissues after freezing. As plants acclimate to low temperatures in autumn, they acquire freezing tolerance and the LT_{50} becomes progressively lower. In breeding programs, plants are often selected for increased freezing tolerance based on changes in LT_{50}; however, any progressive change that occurs during cold acclimation is correlated with the acquisition of freezing tolerance. Therefore, although AFPs accumulate in the apoplast of winter cereals during cold acclimation as the plants acquire freezing tolerance [34], it is still important to establish that AFPs directly influence the freezing process *in planta*.

Determining the role of AFPs has been approached in several ways. Simply increasing the AFP content of canola (*Brassica napus*) leaves by vacuum-infiltrating a solution of winter flounder type I AFP into the apoplast lowered their freezing temperature by 1.8°C [38], whereas

Table 2. Transgenic plants expressing a gene encoding an antifreeze protein[a]

Gene origin	Plant	Phenotype of transgenic lines	Refs
Fish			
Synthetic winter flounder (*Pa*) type I AFP fused with truncated staphylococcal protein A	Tomato (*Lycopersicon esculentum*)	Recombinant protein accumulated within cell and inhibited ice recrystallization	[41]
Winter flounder (*Pa*) type I AFP (preproAFP cDNA)	Tobacco (*Nicotiana tabacum*)	Recombinant proAFP accumulated at 4°C but not at 25°C	[47]
Synthetic winter flounder (*Pa*) type I AFP fused with signal peptide of common bean phytohemagglutinin	Potato (*Solanum tuberosum*)	Recombinant protein accumulated, lowered LT_{50} by 1°C for 3 h	[44]
Sea raven (*Ha*) type II AFP fused with the signal peptide of tobacco *PR-1b*	Tobacco (*Nicotiana tabacum*)	Recombinant protein accumulated in apoplast, exhibited 0.01°C thermal hysteresis, inhibited recrystallization with no effect on LT_{50}	[43]
Insect			
Synthetic spruce budworm (*Cf*) AFP fused with the signal peptide from tobacco *PR-1b*	Tobacco (*Nicotiana tabacum*)	AFP accumulated in apoplast, exhibited 0.37°C thermal hysteresis and inhibited ice recrystallization	[42]
Fire-colored beetle (*Dec*) AFP	*Arabidopsis thaliana*	AFP accumulated in apoplast and exhibited 0.42°C thermal hysteresis; after nucleation, lowered freezing temperature of whole plants by 1.3°C with no effect on LT_{50}	[46]
Plant			
Carrot (Dac) *AFP*	Tobacco (*Nicotiana tabacum*)	AFP accumulated in apoplast, inhibited ice recrystallization and produced 0.35 to 0.56°C of thermal hysteresis	[23]
Carrot (Dac) *AFP*	*Arabidopsis thaliana*	AFP accumulated in apoplast and modified ice crystal morphology	[22]
Carrot (Dac) *AFP*	*Tobacco* (*Nicotiana tabacum*)	Survived supercooling to −2°C	[45]
Winter rye (Sc) chitinase-AFP	*Arabidopsis thaliana*	AFP accumulated in apoplast with no effect on LT_{50}	[b]

[a]Abbreviations: AFP, antifreeze protein; Cf, *Choristoneura fumiferana*; Dec, *Dendroides canadensis*; Dac, *Daucus carota*; Ha, *Hemitripterus americanus*; Pa, *Pseudopleuronectes americanus*; Sc, *Secale cereale*.
[b]D.L. Buhlers, pers. commun.

extracting AFPs from cold-acclimated winter rye leaves did not affect the freezing temperature but did increase injury after freezing and thawing [34]. Winter rye AFPs had no effect on protoplast survival after freezing and thawing, but did lower the LT_{50} of nonacclimated winter rye suspension cells by 2.5°C [36]. Because winter rye AFPs are associated with the outer surfaces of cell walls [35,40], they can reduce freezing injury in intact cells by inhibiting the propagation of ice through the cell wall or by binding to extracellular ice and slowing its growth [39].

Plant transformation with genes encoding antifreeze proteins

Agricultural production in many areas is limited by freezing temperatures. Higher yields could be achieved either by improving the freezing tolerance of an overwintering crop or by increasing the survival of freezing-sensitive crop plants following light frosts. Moreover, AFPs could increase the shelf life and improve the quality of frozen foods by inhibiting the recrystallization of ice if the AFPs are targeted to accumulate in fruits and vegetables before harvest [41]. Therefore, it is not surprising that easily transformed plants, such as tobacco, tomato, potato and *Arabidopsis*, have been used to study the effect of expressing cDNAs encoding AFPs originally isolated from fish, insects or plants (Table 2). To date, most transformation experiments have focused on ensuring that the transgene is expressed and that its product is functional and accumulates in the apoplast. In the case of fish and insect AFPs, the nucleotide sequences were synthesized to ensure the use of codons preferred by the host plant and the transformation vectors have included a signal peptide targeting the protein for secretion [41–44]. When plants transformed with genes encoding fish, insect or plant AFPs were cooled in the presence of an ice nucleator, there was no change in LT_{50}. However, in the absence of an ice nucleator, plants transformed with genes encoding AFPs supercool by 1–3°C more than wild type [43–46].

Although the CaMV 35S promoter is used to achieve constitutive gene expression in all plants transformed to produce AFPs, cold acclimation still has an effect on the accumulation and/or activity of the AFPs. For example, the mRNA and its corresponding AFP from winter flounder accumulate in transgenic tobacco only at low temperatures because turnover of the transcript occurs more slowly and the secondary structure of the protein is more stable [47].

Conclusions

Plant AFPs are unusual proteins: they have multiple, hydrophilic ice-binding domains that appear to function as inhibitors of ice recrystallization and ice nucleation. AFPs have little effect on LT_{50} but could enhance winter survival by slowing freezing processes. Moreover, most AFPs from plants are modified PR proteins that retain high sequence identify and even the antifungal activities of the progenitor PR proteins. Therefore, genes encoding plant AFPs are excellent models for use in evolutionary studies to determine how genes acquire new expression patterns and how proteins acquire new activities. Resolving the structures and identifying the ice-binding domains of additional plant AFPs will be important not only in understanding protein–ice interactions, but also in creating novel ice-binding domains in other proteins. Because multiple proteins are required to inhibit the growth of ice and pathogens, multigene transformation could be required to transfer these characteristics to other plants.

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Supplementary data

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