



Review

# Antifreeze proteins in higher plants

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## Abstract

Overwintering plants produce antifreeze proteins (AFPs) having the ability to adsorb onto the surface of ice crystals and modify their growth. Recently, several AFPs have been isolated and characterized and five full-length AFP cDNAs have been cloned and characterized in higher plants. The derived amino acid sequences have shown low homology for identical residues. Theoretical and experimental models for structure of *Lolium perenne* AFP have been proposed. In addition, it was found that the hormone ethylene is involved in regulating antifreeze activity in response to cold. In this review, it is seen that the physiological and biochemical roles of AFPs may be important to protect the plant tissues from mechanical stress caused by ice formation.

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**Keywords:** Antifreeze protein; Antifreeze cDNA; Ice formation; Overwintering plant

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## 1. Introduction

Plants are poikilotherms; that is, they assume the temperature of their environment. The stresses imposed by temperature have important implications for agriculture. For example, it has been conjectured that a 1 °C

decrease in the world mean temperature would result in a 40% reduction in rice production (Hale and Orcutt, 1987). In the past two decades, considerable effort has been directed at understanding the phenomenon of temperature stress and adaptation to temperature in plants (Guy et al., 1985; Thomashow, 1990, 1998, 2001; Urrutia et al., 1992; Hughes and Dunn, 1996; Griffith et al., 1997; Hoshino et al., 1999; Jia and Davies, 2002). Since temperature is a major uncontrollable climatic

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factor, more research is needed on inheritable characteristics for temperature stress resistance and on the manipulations of plant physiology to increase tolerance of temperature extremes.

Temperature stress of plants can be divided into the effects of temperature that cause high temperature, chilling and freezing injuries (Hale and Orcutt, 1987). Plants differ in their resistance to chilling and freezing temperatures. The two mechanisms for freezing resistance are avoidance and tolerance (Lewitt, 1980; Franks, 1985; Ashworth and Kieft, 1995). Freeze-avoiding plants may avoid freezing by overwintering as a seed with little freezable water, or they may completely avoid any freezing in the tissues by supercooling and/or lowering the freezing point by antifreeze substances. Freezing tolerance is the capacity to withstand extracellular ice formation and to avoid intracellular ice formation (Zamecnik and Janacek, 1992). Extracellular freezing produces a freeze-dehydration due to removal of water from the cytoplasm to the growing ice crystals. As freeze-dehydration proceeds, the cell contents become more and more concentrated (Lewitt, 1980). Therefore freezing-tolerant plants must either avoid or tolerate freeze-induced cellular dehydration (Antikainen, 1996). Freezing-tolerant plants exhibit injury only at temperatures lower than the temperature at which extracellular ice formation begins (Antikainen, 1996). Plants produce several compounds to protect cells against fatal intracellular and intercellular ice formation. Many overwintering plants accumulate sugars, amino acids and antifreeze compounds including antifreeze proteins (Griffith et al., 1997; Thomashow, 1998, 2001; Hoshino et al., 1999; Ewart et al., 1999; Yu and Griffith, 2001).

In this review, we introduce antifreeze proteins (AFPs) and their biological importance in overwintering higher plants.

## 2. Low temperature and plants

In winter, the temperature in cold areas sometimes drops to below  $-40^{\circ}\text{C}$ . Overwintering plants found in Arctic, Antarctic and Alpine climates have developed a high level of freezing tolerance. For example, *Silene acaulis* and *Carex firma*, both of which grow at high altitudes in European Alpine regions, can survive at temperatures of less than  $-50^{\circ}\text{C}$  (Sakai and Larcher, 1987). In culture crops, the freezing-tolerant cultivar of winter wheat can survive at temperatures of less than  $-25^{\circ}\text{C}$  (Yoshida et al., 1997) and some feeding grasses can survive at temperatures of less than  $-30^{\circ}\text{C}$  (Moriyama et al., 1995). These findings suggest that certain plants have special mechanisms to protect themselves against freezing stress.

A knowledge of the physical chemistry of water and the freezing process is needed to understand what hap-

pens when plants are subjected to freezing temperatures. The plant consists of the cell interior referred to as the intracellular and the extracellular (apoplast) spaces. Traditionally, plant physiologists have considered water contained in plants to be divided between two main compartments. Symplastic water is that within the plasma membrane and includes all the water in the cytoplasm and the central vacuole. The apoplastic water is all the water outside the plasma membranes. The apoplast is divided into three separate spaces: the xylem-lumen apoplast, the cell-wall apoplast, and the intercellular-space apoplast. This includes all water in the cell wall, extracellular spaces, and xylem conduits. Apoplastic water has traditionally been thought of as a dilute solution freely moving in cell walls, between cells, and in the xylem (Canny, 1995). As temperatures drop below  $0^{\circ}\text{C}$ , ice formation is typically initiated in the extracellular spaces of plants because the extracellular fluid generally has a higher freezing point than the intracellular fluid. Freezing is triggered by extracellular ice nucleators, which are materials that cause crystallization of the water. Secreted ice nucleators play an important role in allowing freezing-tolerant plants to survive low or prolonged subzero temperatures by minimizing the extend of supercooling, so that freezing becomes a relatively slow and controlled process (Franks, 1985; Hale and Orcutt, 1987; Ashworth and Kieft, 1995; Thomashow, 1998, 2001; Snider et al., 2000).

In plant tissues, intracellular ice formation is considered to be lethal to the cell because the plasma membrane is irreversibly ruptured resulting in the membrane destabilization and the loss of semipermeable characteristics (Steponkus, 1984). Freezing-tolerant plants must have evolved mechanisms that allow them to avoid intracellular ice formation. Recent studies related to freezing-tolerant plants have elucidated some important physiochemical properties of apoplastic solutes that affect our understanding of ice formation in plants (Griffith et al., 1997; Hoshino et al., 1999; Ewart et al., 1999; Yu and Griffith, 2001). Qualitative and quantitative differences in protein content between non-acclimated and cold-acclimated plants have been reported in various plants (Cloutier, 1983; Griffith et al., 1997; Hoshino et al., 1999; Ewart et al., 1999; Atıcı and Nalbantoğlu, 1999a,b; Yu and Griffith, 2001). Overwintering plants require a period of acclimation to cold temperature to develop freezing tolerance. This process requires the accumulation of proteins whose synthesis increases at low temperature. Some of these proteins are intracellular, including dehydrins, proteins involved in carbohydrate metabolism, 14-3-3 proteins, kinase regulators, and cryoprotective proteins (Jarillo et al., 1994; Close, 1997; Wisniewski et al., 1999). The functional characteristics of the cryoprotective proteins are the protection of intracellular proteins and membrane during the freeze-thaw process (Anchordoguy et al., 1987).

It was reported that  $\beta$ -1,3-Glucanase, cryoprotective protein, protects thylakoids against freeze–thaw injury (Hinch et al., 1997). In addition, 3 types of proteins accumulate outside the cells during cold acclimation. These include cell wall-modifying proteins (Showalter, 1993), pathogenesis-related (PR) proteins (Hiilovaara-Teijo et al., 1999) that protect the plants against disease-causing organisms, and antifreeze proteins (AFPs) that interact with ice (Griffith et al., 1992, 1997; Hoshino et al., 1999; Ewart et al., 1999; Yu and Griffith, 2001).

### 3. Identification and characterization of antifreeze proteins

AFPs have been discovered in many freezing-tolerant organisms, including fish, insects, terrestrial arthropods, bacteria, fungi and plants (Duman and Olsen, 1993; Duman et al., 1993; Griffith and Ewart, 1995; Griffith et al., 1997; Hoshino et al., 1999; Ewart et al., 1999; Yu and Griffith, 2001). In some studies related to plants, winter and spring rye, winter and spring wheat, winter and spring canola, winter barley, spring oat, cabbage, maize, spinach, kale, carrot, *Ammopiptanthus monolicus*, *Solanum dulcamara*, *Lolium perenne* and tobacco have been chosen to investigate AFPs (Griffith et al., 1992, 1997; Marentez et al., 1993; Hon et al., 1994; Antikainen and Griffith, 1997; Atıcı and Nalbantoğlu, 1999a,b; Meyer et al., 1999; Yong et al., 2000; Huang and Duman, 2002; Pudney et al., 2003). A different approach to elucidate the identity, function and location of AFPs was chosen by Griffith et al. (1992). They started to look for proteins associated with

increased freezing tolerance in the intercellular spaces where ice formation occurs. Some specific proteins have been purified from the apoplastic region of winter and spring rye, winter and spring wheat, winter barley and spring oats leaves after cold acclimation. It has been shown that these proteins exhibit antifreeze activity causing bipramidal ice crystal growth (Griffith et al., 1992; Marentez et al., 1993; Hon et al., 1994; Antikainen and Griffith, 1997).

The effect of apoplastic solution on freezing injury was investigated on winter wheat and winter rye plants with cold-acclimated (Marentez et al., 1993; Atıcı and Nalbantoğlu, 1999b). After an apoplastic solution had been extracted from the leaves, freezing injuries in extracted leaves were increased (Fig. 1). These experiments demonstrated that the apoplastic solution including apoplastic proteins do reduce the injury (Atıcı and Nalbantoğlu, 1999b).

The effect of apoplastic proteins on ice nucleation temperature was also investigated on winter wheat and winter rye plants with cold-acclimated (Marentez et al., 1993; Atıcı and Nalbantoğlu, 1999b). The apoplastic proteins were precipitated from apoplastic solutions extracted from cold-acclimated and control leaves. The ice nucleation temperatures determining with the apoplastic proteins in cold-acclimated leaves were lower than in control leaves (Fig. 2). These experiments demonstrated that the apoplastic proteins do reduce ice nucleation temperature (Atıcı and Nalbantoğlu, 1999b).

Thermal hysteresis activity (the difference between freezing and melting points characteristic of the presence of AFPs) can provide a quantitative assay for

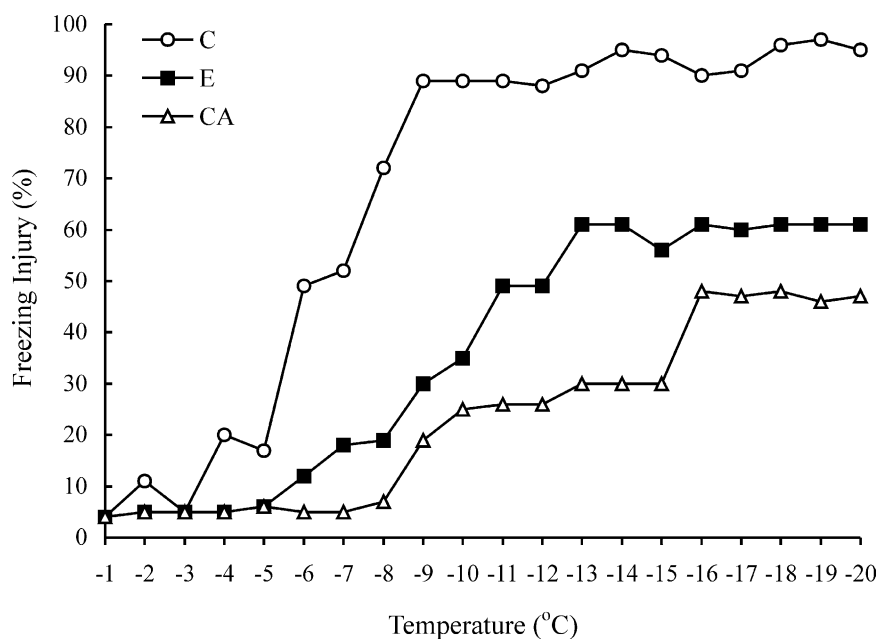


Fig. 1. Effect of apoplastic solution on freezing injury in winter wheat leaves at different conditions (Atıcı and Nalbantoğlu, 1999b). C: control leaves growing at 20/18 °C, CA: cold-acclimated leaves growing at 5/2 °C, E: leaves in which solution of apoplastic region was extracted from CA leaves.

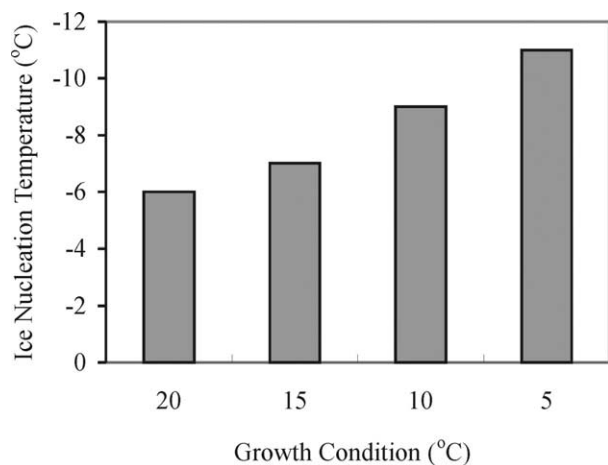


Fig. 2. Effect of apoplastic proteins on ice nucleation temperature (Atıcı and Nalbantoğlu, 1999b). Apoplastic proteins were obtained from winter wheat leaves at different growth conditions. 20: apoplastic proteins were obtained from control leaves growing at 20, 15, 10 and 5 °C; apoplastic proteins were obtained from cold-acclimated leaves growing at 15, 10 and 5 °C.

AFP activity (DeVries, 1986). Antifreeze activity is also assayed qualitatively by examining the morphology of ice crystals grown in the AFPs. However, the latter may be more useful in plants because the levels of thermal hysteresis in plants are relatively small, compared to that in fish or especially insects. Antifreeze activity of apoplastic proteins obtained from winter and spring rye, winter and spring wheat, winter barley and spring oats leaves was qualitatively investigated (Griffith et al., 1992, 1997; Marentez et al., 1993; Hon et al., 1994; Antikainen and Griffith, 1997; Yu and Griffith, 2001).

The crystal shapes are dependent on AFP concentration and specific activity. In the absence of AFP, an ice crystal grows into the shape of a flattened disc (Fig. 3A). At low AFP concentrations (nM), they adsorb to the prism face of the crystal surface, resulting in the growth of a hexagonally shaped ice crystal (Fig. 3B). At high AFP concentrations (μM), they are still hexagonal, but are also elongated to form columns or bipyramids (Fig. 3C). At higher AFP concentrations above 100 μM, the ice crystals form elongated needle-like shapes (Fig. 3D) (Griffith et al., 1992, 1997; Hoshino et al., 1999; Meyer et al., 1999; Yu and Griffith, 2001).

Plant proteins demonstrating antifreeze activity have been isolated and characterized from the following plants: *S. dulcamara* 67 kDa (Duman, 1994), winter rye 11–37 kDa (Hon et al., 1994, 1995), kale 66 kDa (Huang and Duman, 1995), peach 60 kDa (Wisniewski et al., 1999), carrot 36 kDa (Worrall et al., 1998; Smallwood et al., 1999), winter wheat 12–39 kDa (Chun et al., 1998; Atıcı and Nalbantoğlu, 1999a), *Arachis hypogaea* 33 kDa (Dave and Mitra, 1998), *Picea abies* 70 kDa (Sabala et al., 1996), *Rhodiola algida* 29–85 kDa (Lu et al., 1998, 2000) and *L. perenne* 29 kDa (Pudney et al., 2003).

AFPs in winter rye have been extensively researched by Griffith and her collaborators (Griffith et al., 1992, 1997; Griffith and McIntyre, 1993; Hon et al., 1994, 1995; Antikainen, 1996; Griffith and Antikainen, 1996; Antikainen and Griffith, 1997; Yu and Griffith, 2001). Seven major polypeptides are visible following SDS-PAGE of apoplastic extracts of cold acclimated winter rye leaves. Five of these apoplastic polypeptides, ranging in size from 16 to 35 kDa, exhibit antifreeze activity (Hon et al., 1994). Previous reports have described the amino acid

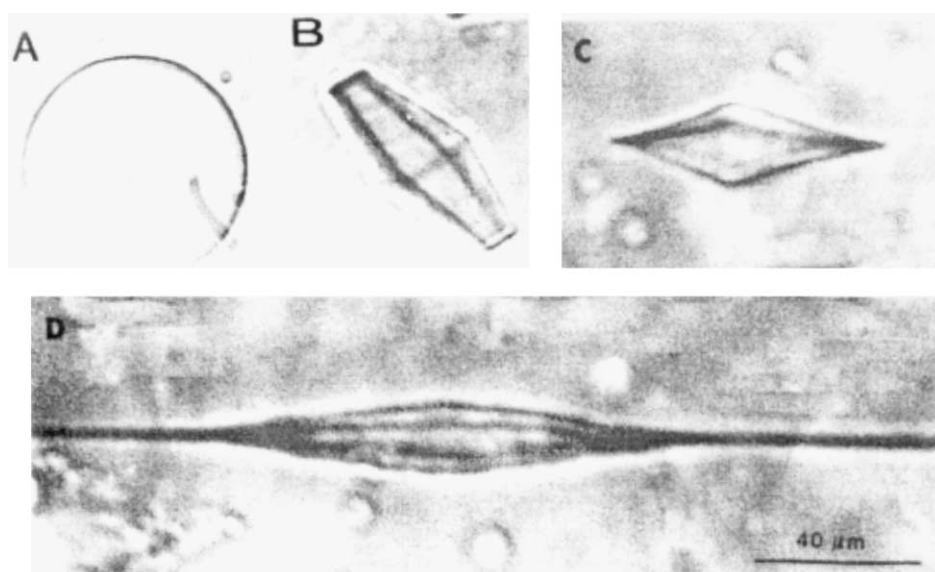


Fig. 3. Effect of winter rye antifreeze proteins on the growth of an ice crystal in solution (Griffith et al., 1992, 1997). (A) An ice crystal grown in water; (B) an ice crystal grown at low AFP concentrations, nM; (C) an ice crystal grown at high AFP concentrations, μM; (D) an ice crystal grown at higher AFP concentrations, above 100 μM.

sequences of winter rye (Hon et al., 1995; Antikainen and Griffith, 1997). Analysis of amino-terminal sequences and immunoblotting of them revealed that these polypeptides are all similar to plant pathogenesis-related (PR) proteins (Hon et al., 1995). In another study related to winter rye, AFPs in their native forms were characterized as oligomeric complexes in vivo (Yu and Griffith, 1999). Nine proteins were separated by native-PAGE from apoplastic extracts of cold-acclimated winter rye leaves. Seven of these proteins exhibited multiple polypeptides when denatured and separated by SDS-PAGE. The complexes of AFPs inhibited ice growth and recrystallization more effectively than the individual polypeptides (Yu and Griffith, 1999).

Antifreeze proteins have the ability to adsorb onto the surface of ice crystals and modify their growth (DeVries, 1986; Knight et al., 1991; Sicheri and Yang, 1995; Antikainen, 1996). The interaction between AFPs and ice crystals has significant effects on the overall growth of ice. Firstly, AFPs inhibit ice crystal growth and depress the freezing temperature of the solution more than would be expected by colligative effects. This property is known as thermal hysteresis (DeVries, 1986). Secondly, AFPs inhibit the recrystallization of ice, which is the growth of larger ice crystals at the expense of smaller ice crystals (Knight and Duman, 1986). Larger ice crystals increase the possibility of physical damage within frozen plant tissues (Griffith et al., 1997). Finally, AFPs also have the ability to interact with ice nucleators, which may result in either the inhibition or the enhancement of ice nucleation activity (Parody-Morreale et al., 1988; Zamecnik and Janacek, 1992; Duman et al., 1993; Jia and Davies, 2002; Duman, 2002).

#### 4. Cloning and characterization of antifreeze protein genes

cDNA encoding an antifreeze protein from the bitersweet nightshade, winter *S. dulcamara*, was cloned by using a polyclonal antibody (Huang and Duman, 2002). The cDNA was determined as 2137 bp in length, encoding of the 591 amino acids of an antifreeze protein of 64 kDa (Figs. 4 and 5). Northern blots demonstrated that the transcript of the protein was not present in leaves until November and December, suggesting that cold acclimation induces this protein production (Huang and Duman, 2002). It was found that the protein contains a zinc finger motif which is present in WRKY proteins, a family of transcription factors which play a role in regulating expression of pathogenesis-related proteins in plants. A unique feature of the protein is that the C-terminus contains 10 consecutive 13-mer repeats. Such repeats are a common feature of animal antifreeze proteins. In addition, it was shown that the expressed fusion protein has specific DNA-binding ability (Huang and Duman, 2002).

In two different studies, the same nucleotide sequences of a gene and cDNA, totalling 1238 bp in length, encoding 332 amino acids of an antifreeze protein of 36 kDa were determined from carrot (*Daucus carota*) using sequence information derived from the purified protein (Figs. 4 and 5) (Worrall et al., 1998; Meyer et al., 1999). It was shown that the AFP gene contains no introns within the coding region and the AFP gene is unique within the carrot genome by using Southern Blot (Smallwood et al., 1999). Expression of the AFP gene was rapidly induced by low temperatures (Worrall et al.,

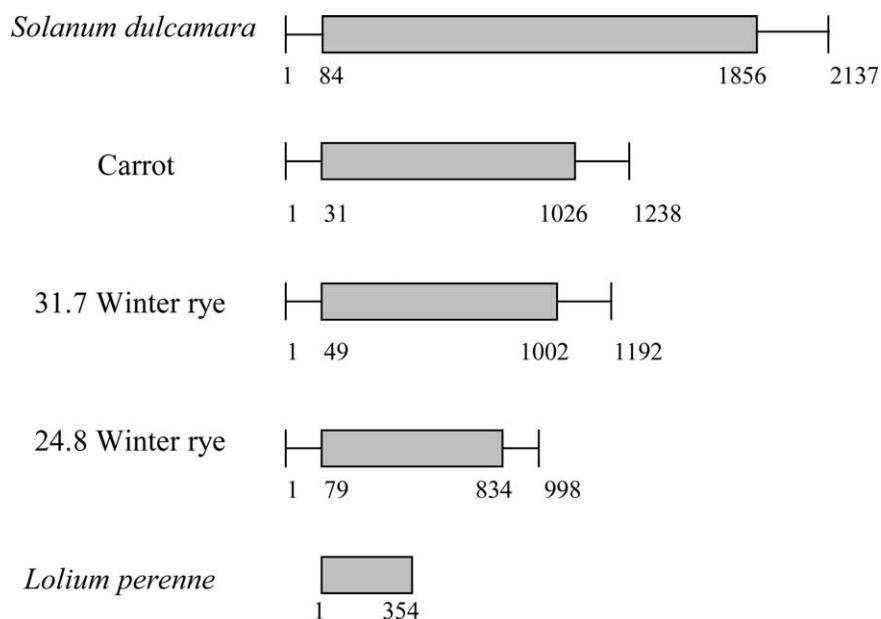


Fig. 4. Diagram of the published cDNAs of plant antifreeze proteins from *Solanum dulcamara* (Huang and Duman, 2002), carrot (Worrall et al., 1998; Meyer et al., 1999), winter rye (Yeh et al., 2000) and *Lolium perenne* (Sidebottom et al., 2000). Boxes represent coding region.



Fig. 5. Multiple sequence alignment of amino acid sequences of the published plant antifreeze proteins. (\*) indicates identical amino acid residues in winter rye (Yeh et al., 2000; GenBank accession no. AF280437 for 31.7 and GenBank accession no. AF280438 for 24.8), carrot (Worrall et al., 1998; GenBank accession No. AAC62932) and *Solanum dulcamara* (Huang and Duman, 2002; GenBank accession No. AF313452). (-) indicates identical amino acid residues in *S. dulcamara* and *Lolium perenne* (Sidebottom et al., 2000; GenBank accession No. AJ277399).

Chitinase genes encoding antifreeze proteins responsive to cold were cloned in winter rye leaves (Yeh et al., 2000). cDNAs coding for two different full-length chitinases were isolated from a cDNA library produced from cold-

cDNA encoding heat-stable antifreeze protein from an overwintering perennial ryegrass, *L. perenne*, was cloned by using the polymerase chain reaction (Sidebottom et al., 2000). The cDNA was determined as 354 bp in length, encoding of the 118 amino acids of an antifreeze protein (Figs. 4 and 5). The protein isolated from its natural source was identified as having an apparent molecular weight of 29 kDa on SDS-PAGE but the 118 amino acid polypeptide sequence is 11.8 kDa. The protein

has six potential *N*-glycosylation sites containing the conserved N-X-S/T glycosylation motif and this therefore explains the discrepancy (Pudney et al., 2003). The protein is very hydrophilic, being rich in asparagine (25%), valine (16%), serine (15%) and threonine (10%), and having very few amino acids with aromatic or hydrophobic side chains. The primary structure has a series of highly conserved, 7-amino-acid repeat sequences with regularly spaced serine and threonine residues. Recently, two different studies have been made about a three-dimensional model for the structure of *Lolium* AFP (Kuiper et al., 2001; Pudney et al., 2003). The first study is a theoretical model based on a beta-roll domain with eight loops of 14–15 amino acids. The fold is supported by a conserved valine hydrophobic core and internal asparagine ladders at either end of the roll. This model displays two putative, opposite-facing, ice-binding sites with surface complementarity to the prism face of ice (Kuiper et al., 2001). The second study is a model based on fourier transform infrared (FTIR) spectroscopy (Pudney et al., 2003). FTIR studies reveal that it has an unusual type of beta-sheeted secondary structure. In addition, in the same study, ice-binding studies of both glycosylated and nonglycosylated expressed forms indicate that it adsorbs to ice through the protein backbone.

The derived amino acid sequences from cDNAs of plant AFPs were shown (Fig. 5). In this review, amino acid sequences of two winter rye, carrot, *S. dulcamara* and *L. perenne* AFPs were compared to see identical residues. It is seen that there is a low homology among these five AFPs although there is 65% homology between 31.7 and 24.8 winter rye AFPs (Table 1). In addition, the review shows that there is 45% homology between repeated sequences at carboxyl end of *S. dulcamara* and repeated sequences of *L. perenne* which is important for ice-binding domains. Therefore, the similarity at carboxyl end of *S. dulcamara* AFP should be taken into consideration for its ice binding domains. Since the amino acid homology is not sufficient to determine ice-binding domains of plant AFPs, in future studies, the ice-binding domains may be perhaps identified by solving the three-dimensional structures of plant AFPs.

There was a report about genetic studies of AFPs and their correlation with winter survival in wheat. It was shown that 5B and 5D chromosomes carry major regulatory genes, which increase both antifreeze activity

and the accumulation of AFPs in plants grown at low temperature (Chun et al., 1998).

## 5. Regulation of antifreeze proteins

How plants sense the change in temperature is not yet known, although evidence for the involvement of  $\text{Ca}^{+2}$  in temperature-sensing and signal transduction has been presented (Minorsky, 1989; Monroy and Dhindsa, 1995; Knight et al., 1996). Recently, the regulation of antifreeze activity was investigated in response to ethylene, salicylic acid, abscisic acid (ABA) and drought in winter rye leaves (Yu and Griffith, 2001; Yu et al., 2001). Nonacclimated rye plants treated with salicylic acid and ABA accumulated apoplastic proteins with no antifreeze activity in rye leaves (Yu and Griffith, 2001; Yu et al., 2001), but, in other two studies, it was found that the secretion of an AFP was induced by ABA in somatic embryos of *P. abies* and in suspension cells of *R. algida* (Dave and Mitra, 1998; Lu et al., 2000). When nonacclimated rye plants were exposed to ethylene and drought both antifreeze activity and the concentration of apoplastic protein increased in rye leaves (Yu and Griffith, 2001; Yu et al., 2001). Rye plants exposed to drought produced ethylene and antifreeze activity in the leaves. It was suggested that ethylene is involved in regulating antifreeze activity in winter rye leaves in response to cold and drought (Yu et al., 2001).

## 6. Physiological and biochemical significance of antifreeze proteins

Some AFPs in winter rye have more than one function. It has been shown that some AFPs are similar to pathogenesis-related (PR) proteins identified as glucanase-like (GLPs), chitinase-like (CLPs) and thaumatin-like proteins (TLPs) in the epidermis (Hon et al., 1995). Winter rye PR proteins have both enzymatic and antifreeze activities (Hon et al., 1995). It was found that two chitinase-AFPs are localized in nonacclimated and cold-acclimated rye leaves (Pihakaski-Maunsbach et al., 2001). In cold-acclimated leaves, chitinase-AFPs were abundant in the walls of epidermal, parenchymal sheath and mesophyll cells and xylem vessels, while less was present in walls of vascular parenchyma cells. In contrast, chitinase-AFP was essentially absent in the nonacclimated cells except in xylem vessels (Pihakaski-Maunsbach et al., 2001). The glucanase-AFPs were observed in the primary cell walls of mesophyll cells adjacent to intercellular spaces, in epidermal cell walls, and in the secondary thickenings of xylem vessel cell walls (Pihakaski-Maunsbach et al., 1996). The glucanase-AFPs also accumulate in the granular material deposited at junctions between adjoining mesophyll cells and in the middle lamella between meso-

Table 1  
Protein sequence identity (%) among plant antifreeze proteins.

	<i>S. dulcamara</i>	Carrot	31.7 W. rye	24.8 W. rye
Carrot	30			
31.7 W. rye	27	29		
24.8 W. rye	27	26	65	
<i>L. perenne</i>	45	ND	ND	ND

ND: lengthy sequence homology was not determined.

phyll cells of winter rye. Winter rye AFPs are present in the cell wall located along pathways for the free movement of water within the plant. The association of AFPs with the cell wall, other cell organelles, and intercellular spaces suggests that these proteins may control the propagation of ice crystals in tissues inward from epiphytic ice nucleators or outward from the vascular bundle, by modifying the crystallization of ice propagators throughout the plant (Griffith et al., 1997; Hoshino et al., 1999; Pihakaski-Maunsbach et al., 2001).

A different property of AFPs is their ability to inhibit ice recrystallisation. This inhibition differs from freezing point depression in that it requires 100–500 times less AFP. The AFPs are believed to inhibit recrystallisation by their adsorption onto ice crystals (Knight et al., 1984). The molecular events that generate ice recrystallization inhibition have not been clearly determined. However, the biological value of this effect is significant (Ewart et al., 1999). Most overwintering plants form ice in intercellular spaces, where AFPs change ice morphology to a weak spike. This phenomenon might act to protect the plant tissues from mechanical stress caused by ice formation (Hoshino et al., 1999).

AFPs purified from winter rye and carrot contain putative signal peptide sequences, suggesting that these AFPs are secreted and primarily function in the extracellular space. However, *S. dulcamara* has a type AFP that does not contain a signal peptide sequence and is therefore likely to remain intracellular. The function of intracellular AFPs is currently unknown. However, it is possible that cytoplasmic AFPs might function to prevent lethal intracellular freezing by inhibiting inoculation from extracellular ice and/or by inhibiting intracellular ice nucleators (Huang and Duman, 2002). If *S. dulcamara* AFP does function as a transcription factor then it should be targeted to the nucleus where it could serve an antifreeze function by inhibiting ice nucleators like other previously described plant AFPs. *S. dulcamara* AFP has sequence homology to other known plant proteins (in this case the WRKY transcription factors) and it demonstrates another potential function (specific DNA binding activity). Consequently, at this time the function(s) of the intracellular *S. dulcamara* AFP is uncertain, not unlike the situation with other plant AFPs (Huang and Duman, 2002).

## 7. Conclusion

The mechanisms by which AFPs in plants inhibit the growth of ice crystals have been elucidated. There have been a few reports on the relationship between the mechanisms of ice growth inhibition and molecular characteristics of plant AFPs, but the molecular interaction between plant AFPs and ice are not well resolved. However, it was assumed that the ice-binding residues of

AFPs must form hydrogen bonds with ice and hydrophobic groups might also contribute to their binding to ice (Sonnichsen et al., 1996; Ewart et al., 1999). Therefore, sequencing and structural studies on AFPs and their genes will be of great interest. By integrating different areas of AFP research, new insights may be gained into their activity and their role in freezing resistance in higher plants. The expression of AFPs in transgenic plants that possess the ability to cold acclimate will allow to test further hypotheses: first, the ability of AFPs to cooperate with endogenous frost protection mechanisms and second, the capacity of AFPs to protect non-acclimated plant tissues against freezing damage. For the second purpose, in recent studies, it was shown that expressions of carrot AFP gene in *A. thaliana* (Meyer et al., 1999) and *N. tabacum* (Worrall et al., 1998) and an insect AFP in *A. thaliana* (Huang et al., 2002) result in an accumulation of antifreeze activity. This kind of research will ultimately allow assessment of the efficacy of the AFPs for the improvement of frost resistance of commercially important crop plant species (Meyer et al., 1999; Ewart et al., 1999).

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