

Antifreeze proteins enable plants to survive in freezing conditions

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Overwintering plants secrete antifreeze proteins (AFPs) to provide freezing tolerance. These proteins bind to and inhibit the growth of ice crystals that are formed in the apoplast during subzero temperatures. Antifreeze activity has been detected in more than 60 plants and AFPs have been purified from 15 of these, including gymnosperms, dicots and monocots. Biochemical characterization of plant antifreeze activity, as determined by the high ice recrystallization inhibition (IRI) activities and low thermal hysteresis (TH) of AFPs, showed that their main function is inhibition of ice crystal growth rather than the lowering of freezing temperatures. However, recent studies showed that antifreeze activity with higher TH also exists in plants. Calcium and hormones like ethylene and jasmonic acid have been shown to regulate plant antifreeze activity. Recent studies have shown that plant AFPs bind to both prism planes and basal planes of ice crystals by means of two flat ice binding sites. Plant AFPs have been postulated to evolve from the *OsLRR-PSR* gene nearly 36 million years ago. In this review, we present the current scenario of plant AFP research in order to understand the possible potential of plant AFPs in generation of freezing-tolerant crops.

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1. Introduction

The magnitude of crop loss due to frost damage is huge and is becoming more severe because of uncertain weather conditions, the result of global warming. Recently, there was unexpected heavy snowfall caused by polar vortex in most parts of North Eastern US, Canada, Europe, Russia and China. The temperature in January 2014 dropped below -50°C in many parts of the US and Canada, which proved detrimental for all life forms and led to significant crop loss. It is estimated that the world's population will reach 9–12 billion in the next 50 years which will require 34–70% more food (FAO 2009, 2010). Therefore, it is quite evident that the growing population and changing environment would

create a huge gap between demand and supply of food in the coming years. One probable way to bridge this gap is to generate freezing-tolerant crops. Although traditional breeding programmes have been able to increase cold hardiness to some extent, these practices have now become impractical due to multigenic cold traits, lack of sufficient genetic markers and limited understanding of mechanism of freezing tolerance in plants. In order to generate freezing-tolerant plants, understanding the mechanism of freezing stress tolerance and identification of potential genes for transfer to cold-susceptible crops is crucial.

Plants can avoid freezing stress by surviving it in a dormant state or they can tolerate it by readjusting their metabolism. The former category of plants is described as

Keywords. Antifreeze activity; freezing stress; ice recrystallization inhibition; thermal hysteresis

Abbreviations used: AFP, antifreeze protein; ECP, extra-cellular protein; IAC, ice adsorption chromatography; IRI, ice recrystallization inhibition; LT, low temperature; TH, thermal hysteresis

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freezing-avoiding, while the latter category is described as freezing-tolerant. Freezing-tolerant plants enhance the capacity of their freezing tolerance when they are exposed to gradually decreasing, but above zero, temperatures by a process known as cold acclimation (Thomashow 1999). During cold acclimation, plants precisely control their physiology and get prepared for facing subzero temperatures by manipulating their gene expression. These freezing-tolerant plants are, therefore, good sources of genes and proteins involved in providing tolerance. These genes/proteins can be manipulated in freezing-susceptible crops to make them freezing-tolerant. One such category of proteins, which was identified in freezing-tolerant plants, is antifreeze proteins. These proteins bind to ice crystals, which are formed preferentially in the apoplast during subzero temperatures, and inhibit their growth. These proteins were originally discovered in an Antarctic marine teleost fish, *Trematomus bernacchii* (DeVries *et al.* 1970; DeVries 1971), and thereafter, these proteins were discovered in almost all the groups of psychrophilic organisms, including plants, insects, fungi and bacteria (Griffith *et al.* 1992; Urrutia *et al.* 1992; Duman and Olsen 1993; Xu *et al.* 1998; Hoshino *et al.* 2003).

Good understanding of the molecular mechanisms of AFP function is required in order to select the candidate AFP gene for making transgenic freezing-tolerant crops. Efforts by the research groups of Marilyn Griffith, JG Duman and PL Davies have brought fruitful results in terms of understanding the biochemical, functional and structural details of plant antifreeze activity (Griffith *et al.* 1992; Urrutia *et al.* 1992; Hon *et al.* 1994; Duman 1994; Hon *et al.* 1995; Xu *et al.* 1998; Hiilovaara-Teijo *et al.* 1999; Kuiper *et al.* 2001; Yaish *et al.* 2006; Yu *et al.* 2010; Middleton *et al.* 2012). The progress of plant AFP research has been reviewed excellently previously (Griffith and Yaish 2004; Atici and Nobtalu 2003; Venketesha and Dayanand 2008); however, since then, significant progress has been made, which needs to be compiled and analysed. In this review we have compiled the recent advances of plant AFP research in order to understand the possible potential of plant AFPs in generation of freezing-tolerant crops.

2. Properties of antifreeze proteins

Antifreeze proteins are a diverse group of proteins that bind to ice crystals and arrest their growth. Detailed characterization of AFPs showed that they exhibit two complementary yet independent properties by virtue of which these are able to protect organisms during freezing stress. These properties are thermal hysteresis (TH) (DeVries *et al.* 1970) and ice recrystallization inhibition (IRI) (Knight and Duman 1986). To understand these properties of AFPs, it is crucial to understand the molecular structure of ice. Ice grows as a hexagonal lattice in which water molecules are held together

by hydrogen bonds. A single ice crystal has two distinct planes – the basal plane and the prism plane (figure 1A). Ice grows due to addition of water molecules to these planes. In the absence of antifreeze activity, ice growth is faster from the prism plane, leading to the formation of wider, disc-shaped ice crystals (figure 1B) (Nada and Furukawa 2005). When ice grows, solute molecules are excluded from the ice lattice, except AFPs/AFGPs, which get irreversibly adsorbed in the growing ice crystals and modify the ice crystal growth. In the presence of antifreeze activity, ice crystals are hexagonal, flower shaped or needle shaped (figure 1C–G). Adsorption of antifreeze activity with ice crystals also results in TH and IRI, which are detailed below.

2.1 Thermal hysteresis

AFPs bind to the ice and prevent the accretion of water molecules to the growing ice crystal planes. This results in a depression of the non-equilibrium freezing point of an aqueous solution below its melting point, causing a difference in freezing and melting points, which is known as thermal hysteresis. Pure water freezes at 0°C at 1 atm; however, due to the presence of various solutes, cell sap freezes at –3°C to –4°C. In the presence of AFPs, the freezing point of cell sap is further decreased, thus avoiding ice formation in plants to some extent, even if everything else is frozen outside. AFPs are 500 times more effective at lowering the freezing temperature than any other known solute molecule (DeVries 1971; Mishra and Pattnaik 1999; Zachariassen and Kristiansen 2000). The depression of freezing point in the presence of AFPs is a non-colligative mechanism as the lowering of freezing point is a thousand-fold higher than expected for the molar concentration of AFPs (Venketesha and Dayanand 2008). During thermal hysteresis, ice is stabilized in form of a supercooled solution. Recently, superheating of ice crystals by AFPs was also shown. These proteins bind to ice crystals and prevent their melting too, a phenomenon known as superheating. However, this phenomenon of superheating is more prominent in hyperactive AFPs (AFPs with higher TH activity, ~2°C at 0.5 mg/mL) than moderate AFPs (AFPs with moderate TH activity, ~0.2°C at 0.5 mg/mL), as shown by one of the plants AFP (*LpAFP* from *Lolium perenne*), which was unable to superheat the ice crystal (Celik *et al.* 2010). In the presence of the most hyperactive AFP (*MpAFP*, AFP from *Marinomonas primoryensis*), superheated ice crystals remained stable for several hours with a maximum superheating of 0.44°C (Celik *et al.* 2010). Although all discovered AFPs possess TH activity, the magnitude of this activity varies in different organisms. Insects have AFPs with the highest TH (3–5°C) followed by fishes (2°C) (Venketesha and Dayanand 2008). The range of TH activity in plants varies from 0 to 2°C. TH depends on the

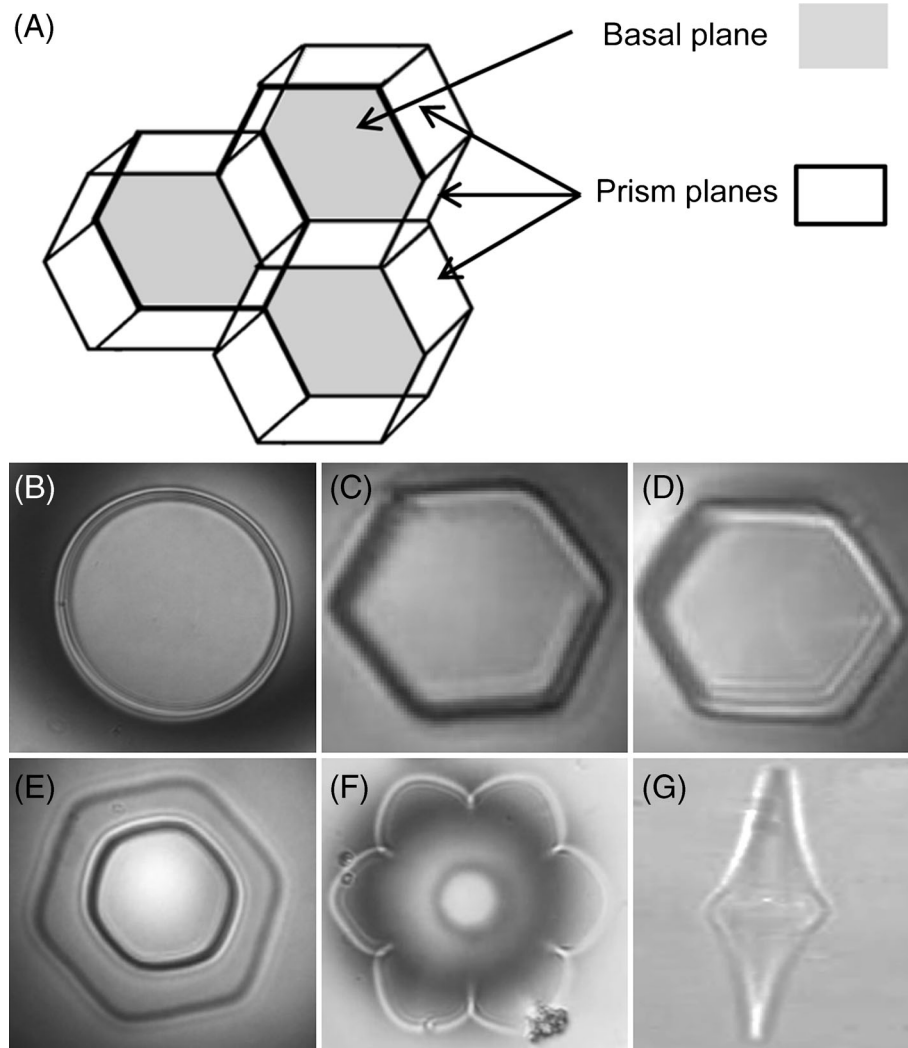


Figure 1. Ice lattice/crystal structure. (A) The structure of ice crystals having basal planes and prism planes. (B–G) Ice crystal morphologies in the absence (B) or presence (C–G) of antifreeze activity.

concentration of AFPs. For various insect and plant AFPs, it was observed that at low concentration of AFPs, there was a steep increase in the TH with increasing AFP concentration. However, at higher AFP concentrations, no further increase in the TH was observed as all AFPs binding sites were occupied (Yeh and Feeney 1996; Zhang *et al.* 2004; Kristiansen *et al.* 2011).

2.2 Ice recrystallization inhibition

Depending upon the type of AFP present, ice formation is inhibited up to a certain temperature, but once ice is formed, another property of AFPs, ice recrystallization inhibition, comes into picture. Ice forms due to crystallization of water

molecules and it grows due to ice recrystallization, i.e. larger molecules of ice grow at the expense of the smaller ones. Larger molecules of ice are lethal as these impose physical stress on the plasma membrane and may rupture it, leading to death of the cell (Steponkus 1984). AFPs inhibit this ice recrystallization and keep a check on the size of ice crystals in order to prevent physical damage. IRI activity is exhibited by the AFPs at concentrations several orders of magnitude below those required for freezing point depression (Venketesha and Dayanand 2008). Plants have AFPs with highest IRI activities.

Interestingly, a recent study showed that TH and IRI are two independent properties of AFPs and that there is no correlation between these two. The experiment was performed using *TmAFP* (AFP from *Tenebrio molitor*), also

called hyperactive AFP due to high TH, and a type III AFP, which is known as moderate AFP due to its low TH activity. It was observed that *Tm*AFP, which produces 10 times higher TH than type III AFP, exhibited 4-fold lower IRI activity in comparison with type III AFP. However, mutations in the ice binding sites resulted in loss of both the activities, suggesting that same ice-binding residues are crucial for both the activities of AFPs (Yu *et al.* 2010).

3. Measurement of antifreeze activity

Both TH and IRI activities of AFPs can be observed and quantified. Nanolitre osmometer and splat assay are the two commonly used techniques to measure these activities. The nanolitre osmometer measures TH activity and provides ice crystal morphology. This instrument consists of a freezing stage coupled with a temperature controller unit. The temperature of the freezing stage can be changed from room temperature to $-20^{\circ}\text{C}/-40^{\circ}\text{C}$ with a precision of $\pm 0.01^{\circ}\text{C}$ within a few seconds. A sample holder disc is mounted on the freezing stage, which consists of small sample loading wells (diameter 0.33 mm). A small volume of mineral oil is applied on the base of the well and allowed to spread in the well by capillary action. Using small capillaries, nanolitre volumes of the sample are suspended in mineral oil. The function of the mineral oil is to prevent dehydration of the sample and also to prevent contact of the sample with the walls of the disc, which otherwise could interfere with the correct measurement of TH activity. After loading, the sample is snap-frozen at $-20^{\circ}\text{C}/-40^{\circ}\text{C}$, and then it is partially melted until only a single ice crystal remains in the well. The temperatures at which this crystal grows and shrinks are taken as the freezing and melting temperatures of the sample, and TH is calculated as the difference between the freezing and the melting points.

Besides the nanolitre osmometer, differential scanning calorimeter (DSC) is also used to determine the TH activity of AFPs. DSC determines the amount of energy required or released to melt or freeze the protein sample as compared to a reference sample. Enthalpy changes in the sample cause a difference in its temperature relative to the reference. Thus, the temperature difference during heating or freezing the samples is recorded and thermal hysteresis is measured as the difference between the melting and freezing points of the sample.

For measuring the IRI activity of AFPs, splat assays and capillary assays are used. Quantification of IRI activity is generally based on the calculation of the endpoint of IRI, which is defined as the lowest concentration of AFPs that still blocks ice recrystallization. Measurement of IRI is the most sensitive method for determination of AFP activity as even a nanomolar concentration of AFPs is sufficient to

inhibit ice recrystallization. However, IRI can also be due to solutes other than AFPs; therefore, IRI assays are generally conducted at high solute concentration (23–30% sucrose) to reduce the non-specific effects. Splat assay was initially demonstrated in 1986 (Knight *et al.* 1986), in which AFP solution (10 μL) is dropped from a 3 m height onto a metal block precooled at -80°C . A thin layer of ice, formed on the metal block, is transferred to a cold stage and allowed to anneal at a temperature just below the melting point of the sample (-6°C to -8°C). After incubation, the ice crystals are observed using a microscope. Later, a modified version of this technique was introduced, which is known as sucrose sandwich or modified splat assay (Smallwood *et al.* 1999). In the sucrose sandwich splat assay, AFPs, dissolved in 23–30% sucrose solution, are sandwiched between two round cover slips (12–18 mm diameter). This sandwich is snap-frozen in an organic solvent (2,2,4-trimethylpentane, *n*-heptane, etc.) at -80°C and then transferred to the same solvent maintained at -6°C to -8°C . After incubation (30 min to overnight), the changes in ice crystal sizes are observed using a microscope. This technique allows unequivocal identification of IRI activity (Smallwood *et al.* 1999). However, with this technique, only one sample can be viewed at a time, and many samples cannot be analysed and compared simultaneously. In addition, the samples cannot be stored for future use. Keeping these limitations in mind, a capillary assay was devised. In this assay different samples are loaded in small capillaries, which are aligned next to each other to view 10–15 samples simultaneously under the same field of the microscope. Also, once the samples are loaded in the capillaries, they can be stored up to 4 weeks without any protein degradation and analysed afterwards (Tomczak *et al.* 2003).

4. Plant antifreeze activity – Current scenario

After the publication of first report on plant AFPs in 1992, antifreeze activity has been detected in more than 60 species (supplementary table 1) and has been purified from 15 of these (table 1). Antifreeze activity in plants can be due to proteinaceous or non-proteinaceous molecules. Protein-based antifreeze activity is either due to antifreeze proteins (AFPs) or antifreeze glycoproteins (AFGPs), while non-proteinaceous antifreeze activity is due to antifreeze glycolipids. AFGPs have been isolated from five plants including *Solanum dulcamara* (Duman 1994), *Daucus carota* (Worrall *et al.* 1998; Smallwood *et al.* 1999), *Lolium perenne* (Sidebottom *et al.* 2000; Pudney *et al.* 2003), *Ammopiptanthus mongolicus* (Yong *et al.* 2000; Fei *et al.* 2008) and *Hippophae rhamnoides* (Gupta and Deswal 2012). The sugar moiety of fish AFGPs interacts with ice. Therefore, to investigate the role of sugars in ice binding, AFGP of *S. dulcamara* was treated with periodate and

Table 1. A summary of physico-chemical properties of purified plant AFPs/AFGPs

S.No.	Plant	Material	MW (kDa)	Cellular localization	Homology	Properties	Reference
1	<i>Ammodramanthus mongolicus</i>	Crude extract from leaves	40, 200 and 39	Cytoplasmic	Agglutinin	Glycosylated, heat stable, 0.9°C TH	Fei <i>et al.</i> 1994; Yong <i>et al.</i> 2000; Fei <i>et al.</i> 2008
2	<i>Bromus inermis</i>	Cell culture	33	Secreted/Apoplastic	Class I chitinase	calcium independent antifreeze activity	Nakamura <i>et al.</i> 2008
3	<i>Chimonanthus praecox</i> L.	Corolla	33	Apoplastic	Class I chitinase	0.52°C TH	Zhang <i>et al.</i> 2011
4	<i>Daucus carota</i>	Tap Root extract	36	Apoplastic	Polygalacturonase inhibitor protein	N-glycosylated, TH-0.36 at 150 µg/mL	Worrall <i>et al.</i> 1998; Smallwood <i>et al.</i> 1999
5	<i>Forsythia suspensa</i>	Crude extracts from bark and leaves	20	Cytoplasmic	Dehydrins	pH sensitive, optimum pH is 7 and it decreases on either side of the optimal pH	Simpson <i>et al.</i> 2005
6	<i>Hippophae rhamnoides</i>	Shoot	41	Apoplastic	Polygalacturonase inhibitor protein	Glycosylated, heat labile, 0.19°C TH and 9 fold IRI	Gupta and Deswal 2012
7	<i>Raphanus sativus</i>	Leaf	-	Apoplastic	-	-	-
8	<i>Lolium perenne</i>	Tuber and leaf	1.32	Apoplastic	No homology reported	TH in tuber and leaves 0.20±0.03 and 0.18±0.02°C respectively	Kawahara H <i>et al.</i> 2009
9	<i>Picea abies</i>	-	29	Apoplastic	No homology reported	TH and ice nucleation not detected (0.03), boiling stable, N-glycosylated	Sidebottom <i>et al.</i> 2000; Pudney <i>et al.</i> 2003
10	<i>Picea pungens</i>	Leaf	70, 27	Apoplastic	Chitinase	TH 2.19 ± 0.83 at 400 µg/mL	Sabala <i>et al.</i> 1996; Jarzabeck <i>et al.</i> 2009
11	<i>Prunus persica</i>	Bark	27	Apoplastic	Chitinase	TH 2.02 ± 0.40	Jarzabeck <i>et al.</i> 2009
12	<i>Rhodiola algida</i>	Cell culture	60	Cytoplasmic, nuclear and chloroplastic	Dehydrin	0.06°C TH	Wisniewski <i>et al.</i> 1999
13	<i>Secale cereale</i>	Leaf	29–85	Apoplastic	No homology reported	Glycosylated, heat labile	Lu <i>et al.</i> 2000
14	<i>Solomon dulcamara</i>	Stem	15–38	Apoplastic	Endochitinase, endoglucanase and thaumatin like proteins	Six AFPs	Hon <i>et al.</i> 1994; Hon <i>et al.</i> 1995
15	<i>Triticum aestivum</i>	Leaf	67	Cytoplasmic	WRKY transcription factor	High glycine content (23.7 mol %), TH-0.3°C at conc.10-35 mg/mL, glycosylated	Duman 1994
16	<i>Triticum aestivum</i>	Leaf	21.3	Apoplastic	Thaumatin like protein	Heat stable, rich in β-sheet and random coil	Chun <i>et al.</i> 1998; Kontogiorgos <i>et al.</i> 2007

borate, which resulted in the loss of antifreeze activity of this protein, suggesting the role of sugar moieties in ice binding. Furthermore, treatment of SdAFGP with β -galactosidase or *Abrus precatorius* lectin also resulted in the loss of activity, suggesting the pivotal roles of galactose in the antifreeze activity of SdAFGP (Duman 1994). In contradiction with these, AFGPs of other plant do not require a sugar moiety for their activity as even deglycosylated AFGPs exhibited similar levels of antifreeze activity (Gupta and Deswal 2012). SdAFGP is homologous to the WRKY transcription factor and is cytoplasmic (Duman 1994), while AFGPs of *D. carota* and *H. rhamnoides* are homologous to polygalacturonase inhibitor protein (PGIP) and are apoplastic (Smallwood *et al.* 1999; Gupta and Deswal 2012). The molecular weight of plant AFGPs ranges from 29 to 200 kDa (table 1).

AFPs in plants have been isolated from 11 plants and are mostly apoplastic, except for the AFPs from *Prunus persica* and *Forsythia suspensa*, which are intracellular, indicating their alternative roles, probably in inhibition of intracellular ice nucleators (Wisniewski *et al.* 1999; Simpson *et al.* 2005). Immunogold localization of *Prunus persica* AFP showed its localization in the chloroplast and nucleus in addition to the cytoplasm (Wisniewski *et al.* 1999). Immuno-blotting, enzymatic assays and N-terminal sequence analysis of plant AFPs shows that they are homologous to the PR-proteins such as thaumatin-like proteins, class I and class II chitinases and β -1,3-glucanase (Hon *et al.* 1995). In addition, AFPs from *P. persica* and *F. suspensa* showed their homology with dehydrins (Wisniewski *et al.* 1999; Simpson *et al.* 2005). Plant antifreeze activity has been characterized by low TH and high IRI activities (Venketesha and Dayanand 2008). However, the growing body of evidence suggests that plants also have antifreeze activity with high TH. AFP of *Picea* sp. is reported to have a TH of nearly 2°C at a concentration of 400 μ g/mL (Jarzabek *et al.* 2009). In the case of IRI, plant AFPs/AFGPs have shown to exhibit maximum activity. The endpoint of IRI was the least for *Lolium* ice-active proteins (IAP) (3 μ g/mL for IAP 3 and IAP 5, and 0.6 μ g/mL for IAP 2), followed by carrot (1 μ g/mL), *F. suspensa* (6 μ g/mL), *H. rhamnoides* (12 μ g/mL) and *D. antarctica* (15.6 μ g/mL). The molecular weights of the plant AFPs range from 1.32 to 70 kDa.

Until 2010, all the plant antifreeze activity known were proteinaceous in nature. In 2011, a non-protein molecule showing antifreeze activity was identified in *S. dulcamara* (Walters *et al.* 2011). This non-protein molecule was identified as a glycolipid and was purified by ice affinity chromatography. This glycolipid produced a TH of $3.1 \pm 0.4^\circ\text{C}$, which is comparable to the TH of insect AFPs. This is the highest TH activity detected in plants till date. Characterization of this glycolipid molecule from Alaskan beetle *Upis ceramoides* showed that it contains little or no

detectable protein, as analysed by amino acid chromatographic analysis, PAGE, NMR spectroscopy and UV-visible spectrophotometry. Structural analysis of this glycolipid molecule showed that it contains a β -mannopyranose-(1 \rightarrow 4) β -xylopyranose backbone and therefore was also termed as xylomannan glycolipid (Walters *et al.* 2009).

As the main function of plant AFPs seems to be IRI, this newly discovered higher-TH glycolipid in plants definitely enriches the freezing fighting arsenal of plants. These antifreeze glycolipids inhibit the formation of ice (by producing high TH) up to a certain degree, but when the ice is formed, their role of ice recrystallization inhibition comes into play. This glycolipid provides additional antifreezing capacity as it has higher TH than AFPs and has thus enhanced the antifreezing potential of plants.

A total of 47 amino acid sequences for plant AFPs/AFGPs are submitted to the database (supplementary table 2). Prediction of physico-chemical properties of 40 plant AFPs/AFGPs using bioinformatics tools showed that 18 of these were hydrophobic while 21 were hydrophilic as concluded by the percentage of their polar and non-polar residues. One AFP from *S. cereale* contained equal number of hydrophilic and hydrophobic amino acids (Muthukumaran *et al.* 2011). Out of the 40 AFP sequences analysed, 21 showed pIs in the acidic range while the remaining 19 proteins had pIs in the basic range. Calculation of instability index showed that 27 proteins were quite stable while the rest of the proteins were unstable. However, the aliphatic index of most of the proteins was high, suggesting that most of these are thermostable. GRAVY index is a measure of hydrophobicities of amino acids, which is important to predict the crystal forming capacity of a protein. Out of the 40 protein sequences analysed, 27 showed optimum GRAVY indices, suggesting these can be used for X-ray crystallographic analysis and are good candidates to study structural details of plant antifreeze activity (Muthukumaran *et al.* 2011).

5. Regulation of the antifreeze activity

Not much is known about the mechanism of regulation of the antifreeze activity in plants. Some reports suggest involvement of hormones like jasmonic acid and ethylene in controlling the antifreeze activity. Winter rye plants, when exposed to ethephon (ethylene-releasing compound) or ACC (ethylene precursor), resulted in the accumulation of antifreeze activity even in non-acclimated conditions (at 20°C), and this effect was blocked by application of AgNO₃ (an ethylene inhibitor), indicating involvement of ethylene in regulating the antifreeze activity. Interestingly, winter rye plants treated with salicylic acid, abscisic acid (ABA) or pathogen attack (*Microdochium nivale*) also

resulted in the accumulation of same set of proteins (chitinase, β -1,3-glucanase and thaumatin-like proteins) in the apoplast; however, these proteins were devoid of the antifreeze activity (Yu *et al.* 2001; Yu and Griffith 2001; Hiilovaara-Teijo *et al.* 1999). The effect of ABA on antifreeze activity was further observed by applying Fluridone (ABA biosynthesis inhibitor) to cold-acclimated winter rye plants, which did not accumulate antifreeze activity in the apoplast, confirming ABA-independent regulation of antifreeze activity (Yu and Griffith 2001).

These results were also confirmed by transcripts analysis in wheat. Expression analysis of wheat transcripts showed that the expression of *TaIRI-1* (*Triticum aestivum* ice recrystallization inhibition protein-1, accession no. AY968588) and *TaIRI-2* (accession no. AY968589) was independent of ABA and SA treatment. Although, *TaIRI-1* transcripts were accumulated in response to jasmonic acid, expression of *TaIRI-2* was neither induced by jasmonic acid nor by ethylene (Tremblay *et al.* 2005). On pathogen attack, *TaIRI-1* and *TaIRI-2* respond differentially, while *TaIRI-1* expression was independent and *TaIRI-2* showed a slight accumulation after pathogen attack (*M. nivale*). Besides, expression of *TaIRI-1* was also independent of the water stress, salt stress, heat shock and wounding (Tremblay *et al.* 2005). These results suggest that different hormones may be involved in the regulation of antifreeze activity; however, further experiments are required to reach to any conclusion.

Plant AFPs are considered to be dual functioning proteins as some of these are homologous to PR proteins and exhibit both antifreeze and hydrolytic activities (Gupta and Deswal 2014). It was found that the chitinases purified from cold-acclimated winter rye and seabuckthorn exhibit hydrolytic as well as antifreeze activities. Similarly, β -1,3-glucanase also retained their partial hydrolytic activities (14–35%) at sub-zero temperatures in addition to antifreeze activity, indicating involvement of some post-translational event in regulating the activities (Yaish *et al.* 2006). However, as both glycosylation-dependent and glycosylation-independent antifreeze activity were observed in plants, glycosylation alone may not be responsible for the regulation of antifreeze activity (Duman 1994; Gupta and Deswal 2012). Similarly, winter rye chitinase undergoes hydroxylation; however, as tobacco chitinase is also hydroxylated and it does not exhibit antifreeze activity, role of hydroxylation in the regulation of antifreeze proteins is not clear (Yeh *et al.* 2000). Currently, there are not ample reports of analysis of post-translational modifications (PTMs) in AFPs, and therefore, at this point of time it is difficult to conclude the role of PTMs in switching of hydrolytic activity of PR proteins to antifreeze activity. One possibility is that PR proteins might interact with some other regulatory molecules that might affect or switch the activities of PR proteins to antifreeze activity (Griffith and Yaish 2004). The growing body of

evidence indicates that refolding of PR proteins during cold acclimation might be responsible for the acquisition of the antifreeze activity. It was observed in seabuckthorn that two isoforms of class I chitinases (HrCHT1a and HrCHT1b) undergo conformational changes during cold acclimation, which might be responsible for their cold-induced antifreeze activity. Interestingly, a similar kind of refolding was also observed in the presence of calcium, suggesting a role of calcium in increasing the antifreeze activity of these two chitinases (Gupta and Deswal 2014). However, antifreeze activity of class I chitinase of bromegrass was not affected by calcium (Nakamura *et al.* 2008), while total antifreeze activity of winter rye was found to be inhibited with higher concentrations of calcium (Stressmann *et al.* 2004), suggesting that different plant AFPs are regulated in a different manner.

6. Temporal and spatial distribution of antifreeze activity

The expression analysis of different AFPs was carried out in many plants to analyse their temporal and spatial distribution. PGIP (accession no. AJ131340) from carrot accumulates within 2 h after exposure to low temperature (Meyer *et al.* 1999), while *TaIRI-1* accumulates just after the transfer of plants to 4°C and reaches the maximum level after 36 days. *DaIRIP4* (AFP from *D. Antarctica*, accession no. FJ663041) showed 40- to 50-fold accumulation in cold-acclimated leaves (John *et al.* 2009). *LpIRI-a* (AFP from *L. perenne*) increased to 40-fold just after 1 h of cold acclimation and reached 8000-fold after 7 days, while *LpIRI-b* accumulated 7-fold and 1000-fold after 1 h and 7 days of cold acclimation respectively (Zhang *et al.* 2010). However, a low level of *DaIRIP1* (accession no. FJ663038) and *DaIRIP4* were detected in *D. antarctica* plants, grown at 22°C. Similarly, *DcAFP* transcripts were also detected in very low levels in carrot seedlings, suggesting constitutive expression of these genes (Meyer *et al.* 1999).

DaIRIP8, *DaIRIP1* and *DaIRIP4* transcripts expression were higher in roots and showed 60-, 700- and 1000-fold increase respectively after 14 days of cold acclimation. *DaIRIP5* showed only 3-fold increase in roots after 14 days of cold, while transcripts of *DaIRIP* were not affected by cold (Chew *et al.* 2012). PGIP was detected in every part of the carrot plant including tap roots, shoots and roots (Meyer *et al.* 1999). Similarly, *TaIRI-1* gets accumulated in almost every part of the wheat plant studied, including leaves, crown and roots, while *TaIRI-2* accumulated only in leaves (Tremblay *et al.* 2005). *DaIRIP* was detected in both roots and leaves in response to cold acclimation. *DaIRIP1* were detected in both leaves and roots, while *DaIRIP4* and *DaIRIP3* were detected in leaves only (John *et al.* 2009).

However, *LpIRIP1* (accession no. FJ663045) transcripts were accumulated approximately 4-fold in cold-acclimated roots only and were not detected in leaves (Zhang *et al.* 2010). These results show that plant produce different AFPs in different tissues and at different time points to immediately face freezing stress. However, to know whether these AFPs share common structural properties or not, structural analysis of plant AFPs were carried out, which are detailed in the following section.

7. Structure and ice binding sites of plant AFPs

After the discovery of antifreeze activity in plants, research was initiated in this area. As AFPs/AFGPs bind to the ice crystal planes and get incorporated into the ice crystal, these must contain a flat surface that can interact with the growing ice crystal planes. Analysis of structural details of some of the plant AFPs showed that these proteins are rich in β -sheeted conformation. Fourier transform infrared spectroscopy of *L. perenne* AFP (LpAFP) showed that it had an unusual type of highly β -sheeted secondary structure (Pudney *et al.* 2003). Similarly, circular dichroism spectra of AFPs from winter wheat, *A. mongolicus* and seabuckthorn class I chitinases, also showed that these were rich in β -stranded conformation (Fei *et al.* 2000; Kontogiorgos *et al.* 2007; Gupta and Deswal 2014). AFP from *A. mongolicus* was composed of 11% of α -helix, 34% of anti-parallel β -sheets and 55% of random coil (Fei *et al.* 2000). Secondary structure prediction using bioinformatics tools of 27 plant AFPs showed that 15 of these were β -sheeted, 7 were α -helical and 5 were composed of both α -helix and β -sheets (Muthukumaran *et al.* 2011). β -Sheets are relatively flat and therefore provide a better surface for ice binding.

The first theoretical model for plant AFP was proposed in 2001 (Kuiper *et al.* 2001). LpAFP was proposed to have a left-handed β -roll supported by a valine hydrophobic core and two internal asparagine ladders on both the sides of the roll. Two flat, opposite-facing ice binding sites, having conserved asparagine residues, were proposed to be complimentary to the prism plane of the ice crystals (Kuiper *et al.* 2001), suggesting that LpAFP binds to the prism plane of the ice crystal only. These two opposite ice binding faces of LpAFP were later termed as a-side and b-side (Middelton *et al.* 2009). The presence of the asparagine in ice binding sites was also confirmed in carrot AFP (DcAFP) by mutational studies. Replacement of asparagine residues of DcAFP with valine or glutamine resulted in a significant loss of TH activity, while an enhancement of TH activity was observed when phenylalanine or threonine was replaced with asparagine (Zhang *et al.* 2004). A 3-D model, proposed for DcAFP, had 10 β -helix loops containing the 24 amino acid tandem repeat (P—L—L—LS—N—L—G—I).

The role of a-side and b-side of the *LpAFP* in ice binding was further studied by mutational studies. It was shown that a single mutation on the a-side, in which outward-pointing short side chain residues of *LpAFP* were replaced by tyrosine, decreased almost 90% of the total TH activity, while a mutation on the b-side had a lesser effect, concluding that only the a-side is prominently involved in the ice binding (Middelton *et al.* 2009). Recently, using X-ray crystallography, it was observed that *LpAFP* binds to both the prism plane and the basal plane of ice crystals, similar to hyperactive AFPs. However, low TH of plant AFPs may be due to the irregular ice binding sites containing Thr, Ser and Val with several side chains, unlike hyperactive AFPs, which contain two rows of aligned Thr residues at their ice binding sites (Middelton *et al.* 2012).

Since only one plant AFP has been crystallized till date, an attempt was made to predict the 3-D structures of the 47 plant AFPs/AFGPs using homology modelling with the help of 'The Protein Model Portal' (Haas *et al.* 2013). Out of the 47 protein sequences, 3-D models of only 9 proteins could be predicted using homology modelling (figure 2). Analysis of predicted 3-D models showed that 3-D structures of PGIP from carrot, WRKY from *S. dulcamara* and thaumatin-like protein from winter rye were dominated by β -sheets. Glucanases from winter rye were composed of both α -helix and β -pleated sheets, while chitinases from winter rye and bromegrass were predicted to be rich in α -helix and random coil (figure 2).

Since purified and sequenced AFPs shared no homology with each other, these proteins are supposed to have evolved independently several times in different lineages of higher plants. The evolution of plant AFPs is discussed in the following section.

8. Evolution of plant antifreeze activity

AFP has been purified from diverse group of plants, including gymnosperms, dicots and monocots. The growing body of evidence suggests evolution of plant antifreeze activity from PR proteins. Carrot AFP (DcAFP), which was homologous to PGIP, was unable to inhibit polygalacturonase extracted from ripe tomato fruit or from *Aspergillus niger* (Worral *et al.* 1998), suggesting that unlike chitinases and glucanases, DcAFP is not a dual functioning protein. Its sequence similarity with PGIP showed that it may have evolved from PGIP gene by mutations in its active site due to which it lost its function of inhibition of polygalacturonase. Further analysis of DcAFP with yeast two hybrid assays using DcAFP (PGIP) as a bait and fungus (*Alternaria alternata*) polygalacturonase as prey showed that there is no interaction between DcAFP and polygalacturonase (Zhang *et al.* 2006). A sequence comparison of DcAFP with other PGIPs showed substitution of

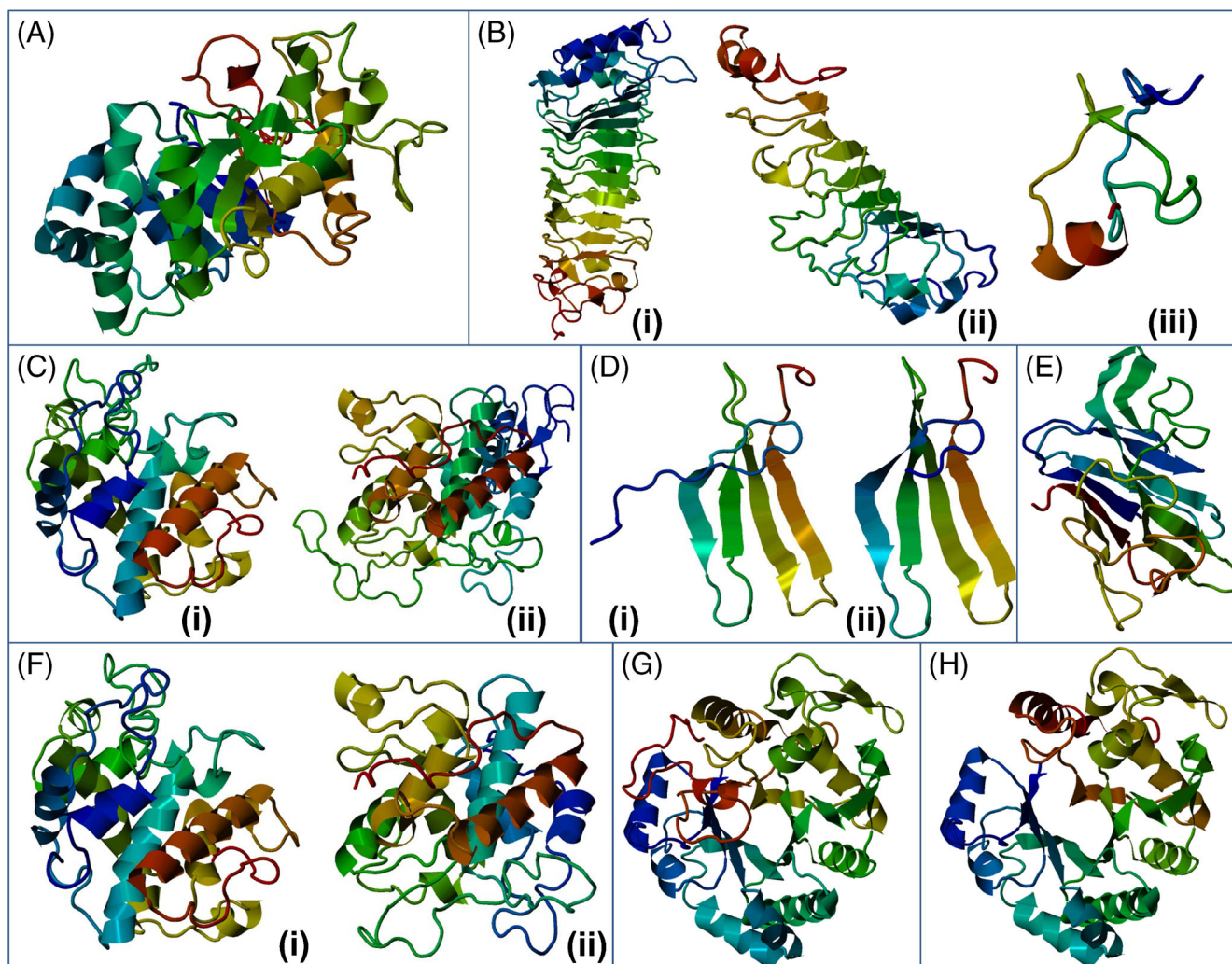


Figure 2. Three-dimensional models of plant antifreeze proteins predicted through homology modelling using ‘The Protein Model Portal’. (A) Model of glucanase 1 of *Secale cereale* from amino acids 24 to 329. (B) PGIP from *D. carota* [a total of 3 models were predicted for this protein from amino acids (i) 27 to 332, (ii) 113 to 300 and (iii) 243 to 288]. (C) Model of class I chitinase from *S. cereale* [a total of 2 different models were predicted for this protein from amino acids (i) 77 to 318, and (ii) 21 to 317]. (D) Model of AFGP from *S. dulcamara* [a total of 2 different models were predicted for this protein from amino acids (i) 361 to 430, and (ii) 191 to 250]. (E) Model of thaumatin like protein of *S. cereale* from amino acids 21 to 173. (F) Model of class II chitinase from *S. cereale* [a total of 2 different models were predicted for this protein from amino acids (i) 23 to 251, and (ii) 19 to 250]. (G) Model of glucanase 3 of *S. cereale* from amino acids 29 to 292. (H) Model of glucanase 2 of *S. cereale* from amino acids 29 to 334. The predicted model for class I chitinase of bromegrass was similar to that of class I chitinase from *S. cereale* and therefore it is not shown in the figure. All predicted models were provided by either ‘SWISS-MODEL’ or ‘MODBASE’.

large number of non-conservative residues with basic amino acids in the β -helix of DcAFP LRR motif, which changes the surface from negative to positive. This could prevent the binding of positively charged DcAFP with positively charged polygalacturonase because of electrostatic repulsion. These results indicate evolution of DcAFP from PGIP gene due to mutations in the non-conservative residues (Zhang *et al.* 2006).

Sequence comparison AFPs isolated from the members of Pooideae subfamily showed the presence of a conserved IRI domain. To explain the evolution of IRI domain in plants, a transposable element (TE) hypothesis was proposed on the basis of sequence homology of its LRR domain with *OsPSR* (*Oryza sativa* phytosulphokine receptor kinase). It was found that the flanking regions of the IRI domain were highly similar to *OsPSR* (Tremblay *et al.* 2005) and therefore

proposed that the formation of IRI domain is a result of TE insertion in the coding region of wheat ortholog of *OsLRR-PSR* (*Oryza sativa* leucine rich repeat-phytosulphokine receptor kinase). After the TE hypothesis, a repeated motif expansion (RME) hypothesis was proposed to explain the evolution of IRI domain as no TE signatures were observed flanking the IRI domain (Sandve *et al.* 2008). According to RME hypothesis, the IRI domain is hypothesized to be evolved by increased copy number of a repeated motif of *OsLRR-PSR*, probably due to illegitimate recombination, slippage or uneven crossing over. As the IRI domain was restricted to the Pooideae subfamily and is not present in all

the members of Pooaceae family, it must have evolved after the divergence of Pooideae from Pooaceae. Therefore, it was estimated that the expansion of the IRI-gene family would have occurred nearly 36 million years ago. This study also suggests the parallel evolution of DcAFP and IRI-domain from *OsLRR-PSR*. The ice binding capacity of DcAFP evolved because of changes in the LRR domain itself, while the IRI domain in grasses seems to have evolved due to the formation of a novel ice binding site from changes in the pre-existing LRR domain (Sandve *et al.* 2008).

In order to shed light on the evolution of AFPs in plants, multiple sequence alignment of all available 47 AFP

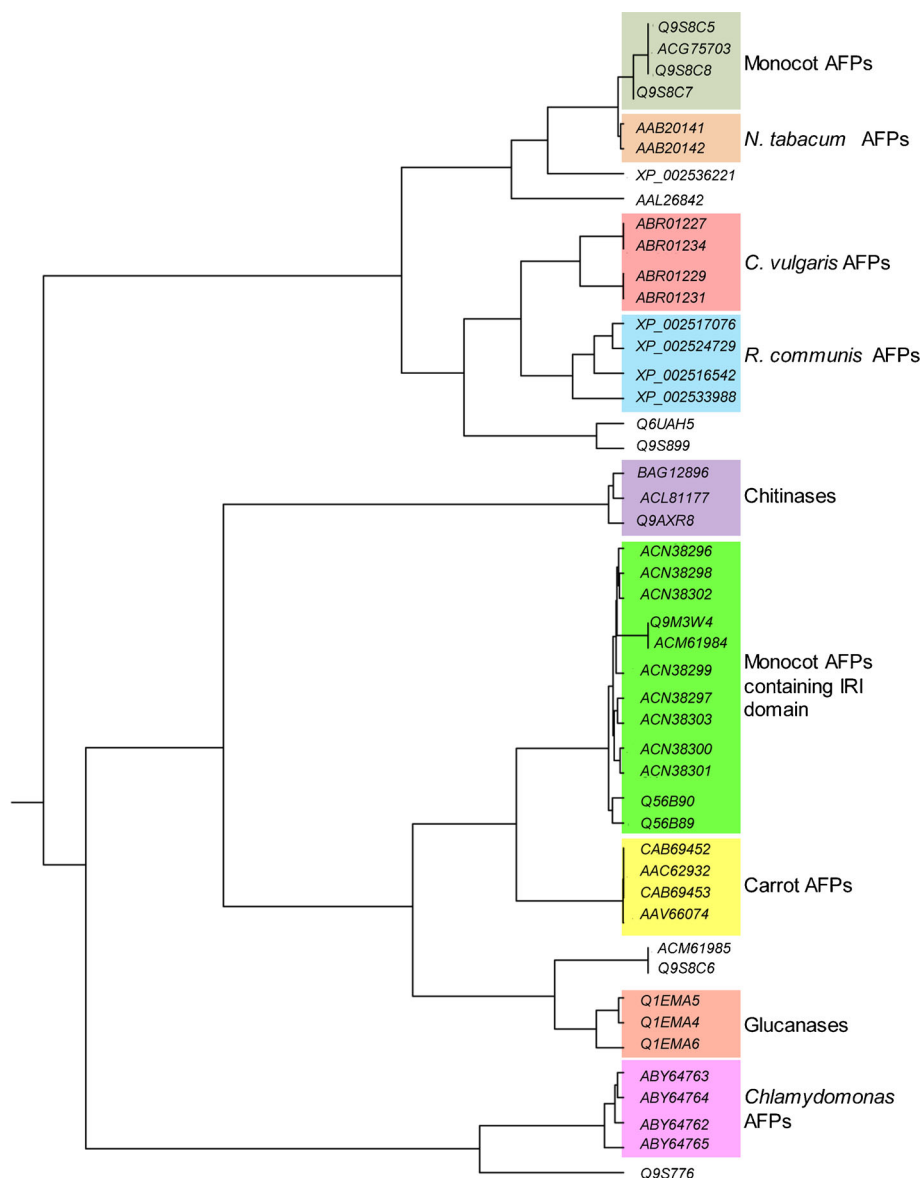


Figure 3. Rooted phylogenetic tree showing the evolutionary relationship among plant AFPs, prepared using the UPGMA method.

sequences were carried out using Clustal Omega (ver. 1.2.1). Results of the alignment showed considerable differences in the available AFP sequences. To analyse the evolutionary relationship among these AFPs, these Clustal Omega results were used to construct an UPGMA (Unweighted Pair Group Method with Arithmetic Mean) rooted phylogenetic tree (figure 3). The phylogenetic tree showed two major clades with multiple sisters and sub-sisters clades. The formation of multiple sisters and sub-sister clades indicate the variations among the AFP sequences. One of the major sub-sister clades with 12 AFPs (shown in green shade in figure 3) was formed from AFPs of the Pooideae subfamily containing the IRI domain. As not much homology in the AFP sequences were found, it can be concluded that different plant AFPs have evolved in an independent manner, except for the AFPs of Pooideae subfamily, which seems to be evolved from a common ancestor.

9. Antifreeze activity and crop improvement

As it is quite established that some of the plant AFPs are dual functioning proteins, these could be the better candidates for crop manipulation. In the past two decades, AFP/AFGP genes have been introduced in some of the economically important plants including potato, tomato and tobacco to make them cold/freezing-tolerant (Hightower *et al.* 1991; Kenward *et al.* 1993; Wallis *et al.* 1997; Worrall *et al.* 1998; Kenward *et al.* 1999; Holmberg *et al.* 2001; Fan, *et al.* 2002). Transfer of the AFP gene in crop plants resulted in enhanced freezing tolerance in many susceptible varieties. Transgenic plants carrying the AFP gene showed inhibition of ice recrystallization and thermal hysteresis (reviewed by Griffith and Yaish 2004). When *DcAFP* was overexpressed in *Arabidopsis thaliana*, accumulation of AFP activity was observed (Meyer *et al.* 1999). Transgenic tobacco

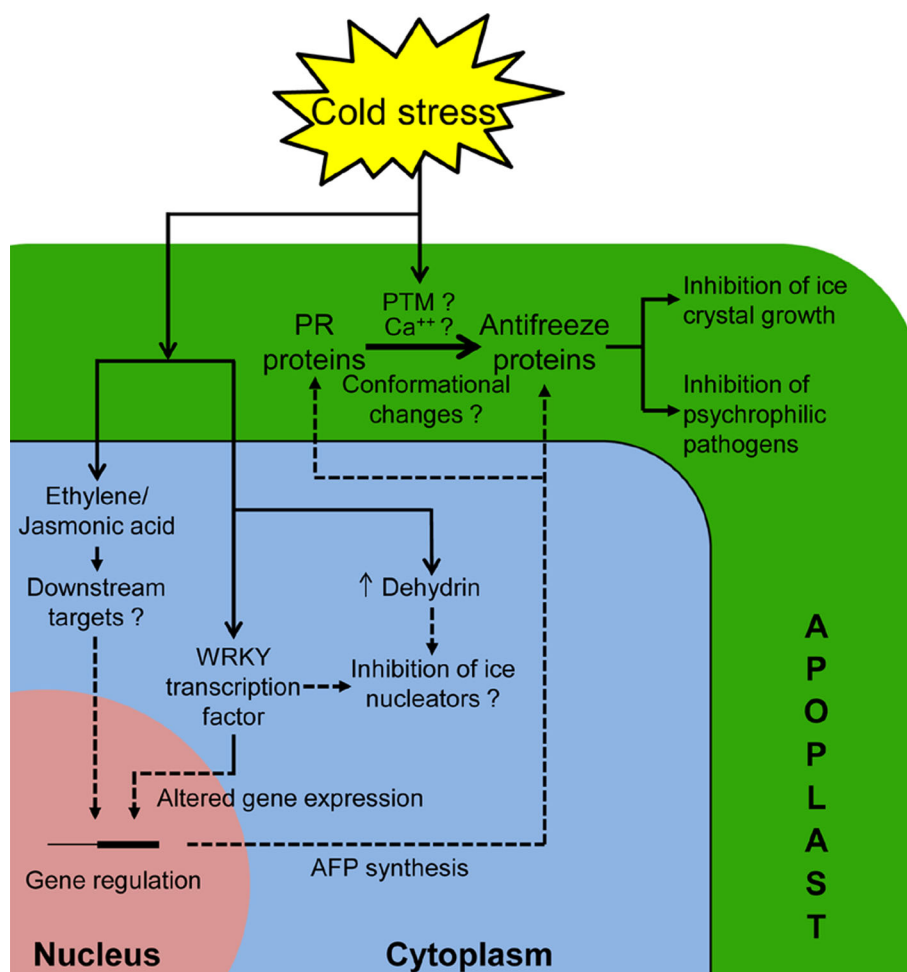


Figure 4. A hypothetical model showing regulation of antifreeze proteins in plants. This model illustrates the function of plant antifreeze proteins in response to cold stress and is generated from the current knowledge of plant AFPs/AFGPs regulation.

overexpressing the carrot AFP gene inhibited ice recrystallization and produced a TH of 0.35–0.56°C. In addition, transgenic tobacco showed improved recovery after cold stress. Ion leakage in these transgenic plants was much less (1–30%) than the wild type (1–80%), suggesting lesser cold-induced membrane damage (Fan *et al.* 2002). Ectopic expression of *LpIRIa* and *LpIRIb* in *Arabidopsis* increased survival rates under both cold-acclimated and non-acclimated conditions (Zhang *et al.* 2010). However, *LpIRIa* was more efficient in providing tolerance to transgenic *Arabidopsis* in comparison with *LpIRIb*. Even freezing tolerance of *E. coli* was enhanced when *LpIRIa/b* was overexpressed (Zhang *et al.* 2010). LT₅₀ is the measure of the effect of cold stress on the plants and their survival ratio. It was observed that transgenic *Arabidopsis* plants having *LpIRI-a/b* showed increased survival rates up to 85–100% compared to wild type (73%) when cold-acclimated at –4°C for 7 days. When these plants were cold-acclimated at –12°C, survival rates of transgenics were changed to 51–78.5% while wild type plants exhibited survival rates of 38.7% (Zhang *et al.* 2010). These results clearly showed that transfer of the AFP/AFGP gene in cold-susceptible plants increases their cold hardiness. Efficacy of AFP genes in transgenic plant can be further increased by transferring a chimeric AFP gene. For example, insertion of a fused gene (spa-afa-5) of AFP III from winter flounder (afa-3 AFP III) and staphylococcal protein-A (spa) in tomato is 10 times more efficient than transfer of afa-3 alone (Hightower *et al.* 1991). It was suggested that the protein-A may be involved in the protection of small AFP from proteolytic digestion.

10. Conclusions

Overall, it is clear that cold stress causes accumulation of AFPs in apoplast of some freezing-tolerant plants. Based on our current knowledge on plant antifreeze activity regulation, a hypothetical model is generated (figure 4). Reports suggested that these apoplastic AFPs are homologous to PR proteins and switch their hydrolytic activity to antifreeze activity during cold stress. However, what is actually responsible for this activity conversion is still unknown. Some reports suggest involvement of calcium in this conversion, while others suggest that the refolding of PR proteins makes these AFPs. However, involvement of any PTM also cannot be neglected. Release of ethylene and jasmonic acid during cold stress is quite evident, and these hormones in turn lead to accumulation of AFPs by changing in gene expression. Besides, expression of WRKY transcription factor also increases during cold stress, which in turn regulates the expression of PR proteins. Gene regulation by ethylene, jasmonic acid and WRKY transcription factor results in synthesis of AFPs, which are secreted in the apoplast.

WRKY transcription factor and dehydrin are the intracellular AFPs that are supposed to inhibit intracellular ice nucleators and thus prevent intracellular ice formation. This conversion of PR proteins to AFP and synthesis of new AFPs results in inhibition of ice crystal growth. As plant AFPs are dual functioning proteins, these also inhibit the growth of psychrophilic pathogens.

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