Ice-binding proteins confer freezing tolerance in transgenic Arabidopsis thaliana

Melissa Bredow1,*, Barbara Vanderbeld1 and Virginia K. Walker1,2

1Department of Biology, Queen’s University, Kingston, ON, Canada
2Department of Biomedical and Molecular Sciences and School of Environmental Studies, Queen’s University, Kingston, ON, Canada

Keywords: Lolium perenne, antifreeze, ice-binding proteins, ion leakage, freezing survival, Arabidopsis thaliana

Introduction

Many overwintering temperate plants are susceptible to freeze injury during the coldest months. At subzero temperatures, ice crystals form in intracellular spaces, or the apoplast, creating an osmotic gradient that can result in cellular dehydration, expansion-mediated lysis of plasma membranes and even death of the plant (Thomashow, 1998). In order to better adapt to freezing temperatures, some overwintering plants induce the expression of a family of protective proteins, designated ice-binding proteins (IBPs). IBPs are members of a highly diverse family of proteins that have been identified in certain organisms including fish (Davies and Hew, 2013), insects (Duman, 2001), bacteria (Gilbert et al., 2004) and plants (Sidebottom et al., 2000). The activity of IBPs stems from their ability to irreversibly adsorb to ice crystals, resulting in the ‘shaping’ of ice as they become incorporated into the ice crystal lattice (Bar-Dolev et al., 2012). IBPs are known to enhance freezing tolerance through two distinct properties: ice recrystallization inhibition (IRI), which prevents the growth of ice crystals at high subzero temperatures (Sandve et al., 2008), and thermal hysteresis (TH), or the depression of the freezing point in relation to the equilibrium melting point (Raymond and DeVries, 1977). While freeze-avoidant organisms produce IBPs that depress the freezing point by several degrees, often referred to as antifreeze proteins (AFPs) (Davies and Hew, 2013; Duman, 2001), plants encode IBPs with low TH activity and rely on restricting ice crystal growth as a primary survival strategy (Sandve et al., 2011).

Although IBPs are not commonly found in plants and are absent in Arabidopsis thaliana for example, they have been identified and purified from more than a dozen plants including bittersweet nightshade (Solanum dulcamara) (Huang and Duman, 2002), carrot (Daucus carota) (Smallwood et al., 1999), winter rye (Secale cereale) (Hon et al., 1995) and perennial ryegrass (Lolium perenne) (Pudney et al., 2003). As ice crystal growth is commonly propagated in the apoplast, secretion of IBPs from the cytoplasm would prevent the recrystallization of extracellular ice, protecting cells from the effects of freeze-induced cellular dehydration. Thus, it is not surprising that most of the IBPs that have been studied have been recovered from the apoplastic extracts of cold-acclimated leaf tissue (Antikainen and Griffith, 1997; Griffith et al., 1992; Hon et al., 1994; Marentez et al., 1993). The presence of an N-terminal signal peptide in most IBPs suggests secretion through the endoplasmic reticulum (ER) secretory pathway. An IBP from S. dulcamara has been reported to lack a signal peptide and remains intracellular (Huang and Duman, 2002), suggesting that IBPs might also function to prevent damage associated with intracellular ice nucleation.

The freeze-tolerant perennial grass, L. perenne (Lp), is native throughout Europe and Eastern Asia where it can survive at temperatures as low as –13 °C (Thomas and James, 1993). Their IBPs have been termed ice recrystallization inhibition proteins (LpIRIPs) due to their low TH activity (~0.3 °C at 1 mg/mL) (Lauersen et al., 2011), but relatively high IRI activity (Sidebottom et al., 2000). Four LpIRIPs have been identified in L. perenne: LpIRI1, LpIRI2, LpIRI3 and LpIRI4 (Sandve et al., 2008). A partial protein product, named LpAFP, was identified in another L. perenne cultivar (Sidebottom et al., 2000) and has been the subject of much in vitro characterization (Lauersen et al., 2011; Middleton et al., 2009).

Summary

Lolium perenne is a freeze-tolerant perennial ryegrass capable of withstanding temperatures below –13 °C. Ice-binding proteins (IBPs) presumably help prevent damage associated with freezing by restricting the growth of ice crystals in the apoplast. We have investigated the expression, localization and in planta freezing protection capabilities of two L. perenne IBP isoforms, LpIRI2 and LpIRI3, as well as a processed IBP (LpAFP). One of these isoforms, LpIRI2, lacks a conventional signal peptide and was assumed to be a pseudogene. Nevertheless, both LpIRI2 and LpIRI3 transcripts were up-regulated following cold acclimation. LpIRI2 also demonstrated ice-binding activity when produced recombinantly in Escherichia coli. Both the LpIRI3 and LpIRI2 isoforms appeared to accumulate in the apoplast of transgenic Arabidopsis thaliana plants. In contrast, the fully processed isoform, LpAFP, remained intracellular. Transgenic plants expressing either LpIRI2 or LpIRI3 showed reduced ion leakage (12%–39%) after low-temperature treatments, and significantly improved freezing survival, while transgenic LpAFP-expressing lines did not confer substantial subzero protection. Freeze protection was further enhanced by the introduction of more than one IBP isoform; ion leakage was reduced 26%–35% and 10% of plants survived temperatures as low as –8 °C. Our results demonstrate that apoplastic expression of multiple L. perenne IBP isoforms shows promise for providing protection to crops susceptible to freeze-induced damage.
LpIRIPs have two distinct domains: a leucine-rich repeat (LRR) domain and a carboxyl (C)-terminal IRI domain, which consists of a series of repeated ‘ice-binding’ motifs (NXXVG/NXXVXG, where X represents an outward-facing residue). The IRI domain of these proteins has been predicted to fold into a β-helix and the crystal structure of LpAFP has verified this fold (Middleton et al., 2009). Specific residues on the ice-binding face (IBF) allow LpIRIPs to adsorb to ice crystals on the basal and primary prism planes resulting in hexagonal bipyramidal crystals (Kumble et al., 2008). N-terminal to the IRI domain is a varying number of LRR motifs, likely derived from phytosulfokine LRR receptor tyrosine kinase sequences (Sandve et al., 2008). The retention of the LRR domain in three of the IBP isoforms may simply have allowed for the presence of the N-terminal signal peptide for secretion to the apoplast.

Notably, one isoform, LpIRI2, lacks most of the region upstream of the IRI domain, including an identifiable signal sequence, and likely evolved through duplication of LpIRI4 and the subsequent deletion of the N-terminal domain (Sandve et al., 2008). The absence of a signal peptide has led to the hypothesis that LpIRI2 is a pseudogene. However, the modest sequence divergence following duplication of LpIRI4 suggests that the gene may still be under selective pressure and thus retain some function (Sandve et al., 2008). Therefore, whether LpIRI2 is a pseudogene, acts as an intracellular IBP or is secreted via a nonclassical pathway is not known.

Biotechnological applications of IBPs for the enhancement of freeze tolerance have been tested in tobacco (Nicotiana tabacum) (Holmberg et al., 2001), potato (Solanum tuberosum) (Wallis et al., 1997) and tomato (Solanum lycopersicum) (Hightower et al., 1991). Generally, such transgenic plants have shown IBP accumulation in the apoplast, but few efforts have reported significant differences in freezing tolerance as shown by 50% lethality (LT50) assays. An exception is the enhanced freeze survival following the transfer of IBP sequences to Arabidopsis thaliana (Zhang et al., 2010). However, these studies have focused on the expression of a single IBP from a protein family, and we considered that the isoforms could work synergistically or cumulatively to restrict ice crystal growth. As well, we were particularly interested in determining whether LpIRI2 has retained ice-binding activity and thus function in planta, despite the loss of the N-terminal signal peptide. We have now addressed these outstanding concerns through the expression of various LpIRIP isoforms in A. thaliana, alone and in combination, in order to provide insight into the mechanisms underlying IBP-mediated freezing tolerance.

Results

Bioinformatics analysis of LpIRIP isoforms

Amino acid alignment of LpIRIP isoforms shows high conservation of the C-terminal residues, while the N-terminal domains are more divergent (Figure 1). The N-terminal domains of LpIRIP isoforms have retained few LRR motifs (0–5 motifs across isoforms), with some isoforms having large deletions, or in the case of LpIRI2, having not retained this domain all together. An N-terminal signal peptide was identified for LpIRI1, LpIRI3 and LpIRI4; however, as previously reported (Sandve et al., 2008), there is no apparent secretion signal within LpIRI2 or LpAFP amino acid sequences. Putative ice-binding amino acids appear to be conserved across four sequences with the unprocessed proteins ranging in size from 151 to 285 residues. The Phyre2 algorithm predicted that all proteins would fold into a right-handed β-helix. While the LpIRI3, LpAFP and LpIRI1 isoforms were predicted to fold into secondary structures with eight β-helical loops, LpIRI2 and LpIRI4 were predicted to have ten loops, as the result of three additional ice-binding motifs (Figure 1).

Functional activity and transcript analysis of LpIRIP sequences

When constructs encoding LpIRI2 and LpIRI3 isoforms were expressed in E. coli, all purified, recombinantly produced proteins restricted ice crystal growth in a splat assay (Figure 2). However, not all recombinant isoforms were equally effective at IRI. High levels of activity were seen with LpAFP and LpIRI3 at 0.01 mg/mL; however, LpIRI2 only demonstrated mild IRI activity, with some ice crystal growth at the same concentration.

Endogenous L. perenne transcript analysis generated ~500-bp and ~850-bp amplification products for LpIRI2 and LpIRI3, respectively. Low levels of LpIRI3 transcript were produced following incubation at 21 °C, but there was no evidence of the LpIRI2 transcript (Figure 3). However, following a 6-d cold acclimation (CA) period at 4 °C both LpIRI3 and LpIRI2 transcripts were abundant.

Ice-binding activity and localization of LpIRIPs in A. thaliana

Following CA, crude cell extracts taken from all four independently generated A. thaliana lines generated LpAFP and LpIRI2 (designated A3), and all three LpIRIP sequences (designated 2A3) showed functional ice-binding activity as demonstrated by IRI analysis (Figure 4a). There was no IRI activity in control CA plants, and very low activity in those transgenic plants kept at room temperature prior to assay (not shown). This was expected as, at least for LpAFP, circular dichroism-monitored conformational changes to the β-helical structure at temperatures >16 °C were coincident with a loss of activity (Lauersen et al., 2011). All CA LpIRI2-transformed lines consistently showed IRI, but they had lower activity than LpAFP- and LpIRI3-expressing lines (Figure 4a). Extracts from the transgenic plants expressing LpIRI2 and LpAFP demonstrated hexagonal ice shaping indicative of adsorption to the primary prism plane (Figure 4b). Notably, LpIRI3, A3 and 2A3 transgenic lines showed hexagonal bipyramidal ice shaping, which is seen with more active AFPs that bind both the primary prism and basal planes. TH activity depends on both ice-binding properties and protein concentration. Indeed, TH values obtained from cell lysates of our transgenic plants were similar for all the single expression lines with the multiple A3 and 2A3 lines showing greater activity levels (Table 1a).

In order to localize IIR activity, the ORFs of LpIRIPs fused to fluorescent markers were expressed in A. thaliana. Confocal microscopy confirmed protein expression in all LpAFP, LpIRI2 and LpIRI3 transgenic lines. It was difficult to resolve cytoplasmic and apoplastic locations however, due to the large vacuoles in leaf and root tissues (Figure S1). Nevertheless, apoplastic extracts of transgenic A. thaliana lines expressing LpIRI2-mOrange and LpIRI3-mOrange showed fluorescence and had high levels of IRI activity (Table 2 and Figure 5a). Additionally, LpIRI2-mOrange lines consistently showed hexagonal, primary prism plane ice shaping and LpIRI3-mOrange lines exhibited hexagonal bipyramidal crystals, consistent with basal and primary prism plane adsorption (Figure 5b). Both LpIRI2-mOrange and LpIRI3-mOrange lines also demonstrated TH activity (Figure 1b). In contrast,
apoplastic extracts from *mOrange*-LpAFP lines had similar levels of ice-binding activity to that of *mOrange* transgenic controls, with low levels of fluorescence (Table 2), no observable IRI activity, ice-shaping activity or TH activity (Figure 5 and Table 1b). To determine whether the *mOrange* tag had disrupted the ice-binding activity of LpIRIPs, the crude cell extracts of transgenic plants were assayed for IRI activity (Figure 6). While *mOrange*-expressing lines had no ice crystal inhibition, all LpIRIP-expressing lines retained high levels of IRI activity. Investigations with guttation fluid of transgenic LpIRIP-expressing lines revealed
similar results to the apoplastic extracts: transgenic LpAFP-expressing A. thaliana and control plants had no detectable IRI activity with LpIRI3 and LpIRI2 lines with high levels of IRI activity (Figure 7). It should be noted, however, that the activity observed in LpIRI2-expressing lines was less than in guttation fluid obtained from LpIRI3 lines (Figure 7).

Electrolyte leakage in transgenic leaves

When leaves were collected from CA transgenic A. thaliana plants and incubated at temperatures slowly ramped down to −6 °C, freezing resulted in the leakage of ions, as assessed by conductivity. The expression of LpAFP in transgenic plants appeared to reduce leaf ion leakage by 6%–10% in three of the four independent lines, but these values were statistically insignificant when compared to control plants (Figure 8a). In contrast, expression of LpIRI3 and LpIRI2 decreased ion leakage by 30%–39% and 12%–22%, respectively (Figure 8b and c).

Transgenic plants expressing multiple LpIRIPs also showed reduced electrolyte leakage compared with controls. A. thaliana leaves from plants expressing A3 constructs showed a 28%–35% decrease in electrolyte leakage following a −6 °C treatment (Figure 9a). Transgenic 2A3 lines similarly showed a 26%–35% reduction in electrolyte leakage (Figure 9b).

LpIRIPs and A. thaliana freeze protection

The addition of the various LpIRIP-bearing sequences dramatically enhanced the freeze survival of whole transgenic A. thaliana plants. Freeze survival was significantly increased compared with controls in two of the four LpAFP-expressing lines following freezing at −6 °C (Figure 10a). In one of the LpAFP lines, there was also a significant increase in survival at −7 °C; however, the overall LT50 was not changed, remaining at −5.6 °C, not significantly different from the LT50 of −5.2 °C seen in control, nontransgenic plants. Significant increases in survival were seen in all four LpIRI3-expressing lines at all tested temperatures between −5 °C and −7 °C (Figure 10b), and this was reflected in a mean LT50 of −6.1 °C. Similarly, LpIRI2-transgenic A. thaliana showed enhanced freeze survival at all temperatures between −5 °C and −7 °C (Figure 10c). In these lines, the mean LT50 was −6.0 °C compared with −5.4 °C in control plants assayed at the same time.

Transgenic plants expressing multiple LpIRIPs also showed enhanced freezing tolerance. Survival was significantly increased in both the A3 and 2A3 lines at temperatures between −5 °C and −8 °C (Figure 11a and b) with a concomitant significant decrease in LT50 to −6.0 °C and −6.4 °C, respectively, compared with the LT50 of −5.2 °C and −5.4 °C for the corresponding nontransgenic controls. Notably, none of the lines bearing a single LpIRI1 sequence or even the A3 lines showed any survival at −8 °C (Figures 10 and 11a), but each of the four 2A3 lines, with all three sequences, showed some survival (10%–17%) at this low temperature (Figure 11b).

Discussion

Freezing tolerance is a complex trait involving biochemical, metabolic and physiological changes. In certain plants, IBPs almost certainly serve as part of a freeze survival strategy to regulate ice crystal growth and to lower the probability of plasma membrane rupture. These proteins have also been shown to lower the activity of bacterial ice nucleation, aiding in freeze survival (Tomalty and Walker, 2014). The L. perenne family of IBPs includes the ‘processed’ protein sequence, LpAFP, which has been extensively characterized in vitro (Lauersen et al., 2011; Middleton et al., 2009); however, the in planta function and activity of the proteins transcribed and subsequently translated...
from the full-length LpIRIPs are less known. Here, our experiments demonstrate that in transgenic A. thaliana, the presence of LpIRI2 and LpIRI3 not only reduced electrolyte leakage but also significantly enhanced freeze survival (Figures 8-11).

Importantly, the degree of freeze protection afforded by LpIRIPs in A. thaliana was correlated with the ice-binding activity localized to the apoplast (Figure 5). Most ice nucleation occurs outside of the cell (Kajava and Lindow, 1993; Xu et al., 1998).

Figure 4 Ice-binding phenotypes in transgenic A. thaliana. Ice recrystallization inhibition analysis of crystals annealed at −4 °C for 18 h (a) and ice crystal morphologies (b) using crude cell lysates collected from control (Col-0) and transgenic plants, including lines expressing LpAFP and LpIRI3 (A3) and all three sequences, LpIRI2, LpAFP and LpIRI3 (2A3). A total protein concentration of 0.1 mg/mL was used for all assays. Only one representative sample is shown for each of the four LpIRIP-expressing lines. All experiments were performed in triplicate.
Intracellular ice nucleation typically only occurs as a result of rapid temperature drops (Siminovitch et al., 1978) and as temperatures were lowered slowly (0.5 °C/h), intracellular ice crystal growth would not be expected in these experiments. In this regard, it may be curious that in two LpAFP-expressing lines, in which the ice activity appeared to have remained intracellular, there was a modest increase in freeze survival, suggesting that intracellular IBPs might additionally contribute to a freeze-tolerant phenotype. Indeed, it is possible that due to the absence of extracellular IBPs and the increased probability of plasma membrane rupture, it has also been suggested that IBPs are capable of preventing cell lysis through physical association with the plasma membranes (Beirão et al., 2011; Hays et al., 1996; Rubinsky et al., 1991; Tomczak et al., 2001). Our results showing reduced electrolyte leakage in transgenic LpIRI2 and LpIRI3 A. thaliana lines are consistent with the interpretation that membrane protection is occurring; however, there is no in planta evidence of a direct association between IBPs and plasma membranes.

Transgenic expression of multiple LpIRIPs reduced ion leakage and increased freeze survival at lower temperatures than in many of the lines bearing a single LpIRIP sequence (Figures 10 and 11). It is thus likely that multiple transgenes result in a greater accumulation of LpIRIPs, even if this was not reflected in TH values given the hyperbolic relationship between TH activity and protein levels. However, the activity of LpIRIPs can be distinguished on the basis of their adsorption to distinct ice crystal planes. Amino acid alignment suggests that the LpIRI3 isoform has the most regular fold, and highest conservation amongst putative ice-binding residues (Figure 1). In accordance with this observation, transgenic plants with the LpIRI3 sequence either as a single copy, with LpAFP, or with LpAFP and LpIRI2, showed adsorption to both the primary prism and basal planes, characteristic of hyperactive AFPs (Pertaya et al., 2008). It is possible that there could be some synergistic activity with the different transgenes, resulting in reduced ice crystal growth when IBPs with slightly different ice plane affinities are combined. As well, IBPs could have differing affinities for any putative membrane-binding sites, which in turn could enhance freeze survival.

These results are reminiscent of the enhancement of TH activity shown when a low-activity type III AFP isoform was expressed in the Notched-fin eelpout along with a high-activity AFP isoform (Nishimiya et al., 2005). It was suggested that AFP isoforms might act cumulatively to enhance activity levels by high-activity AFPs slowing the growth of ice crystals sufficiently so that less active AFPs would have time to adsorb, or alternatively, that less active AFPs could adsorb at ice crystal sites located between the binding sites of more active AFP isoforms (Nishimiya et al., 2005). Similarly, Burcham et al. (1984) hypothesized that antifreeze activity was enhanced in the presence of high- and low-activity antifreeze glycoprotein (AFGP) isoforms, as a result of a cooperative coverage of the initial ice crystal. It is possible that LpIRIPs may function similarly, restricting growth of the initial seed crystal more effectively when more than one isoform is present, providing optimal freeze protection to plants.

Putting aside possible isoform differences in ice or membrane substrate affinity, our results clearly demonstrate that the LpIRI2 isoform with no identifiable signal sequence shows ice-binding activity (TH, ice-shaping and IRI) and CA accumulation in the apoplast, and can confer freeze protection to a host plant. Thus, the evolutionary loss of the N-terminal domain does not render this protein nonfunctional. Similar transcript-level estimates of LpIRI2 and LpIRI3 in CA L. perenne leaves, and the reduction in A. thaliana leaf electrolyte leakage, further support our contention that LpIRI2 is not a pseudogene and could therefore play a role in L. perenne overwintering. As intracellularly localized IBPs are produced in certain plants including the desert evergreen shrub, Ammopiptanthus mongolicus (Fei et al., 1994), the flowering shrub, Forsythia suspense (Simpson et al., 2005), and S. dulcamara (Duman, 1994) as well as the Antarctic microalga, Chaetoceros neogracile (Gwak et al., 2014), we initially suspected that LpIRI2 functioning similarly. Of note, intracellularly localized IBPs in addition to restricting ice crystal growth have demonstrated comparably lower ice-binding activity (Duman, 1994). Nonetheless, our experiments indicate that the LpIRI2 isoform is secreted (Table 2 and Figures 5 and 7). Although levels of IRI and TH activities in the apoplast were admittedly lower than

| Transgenic line          | Thermal hysteresis activity (°C) |
|--------------------------|----------------------------------|
| (a)                      |                                   |
| Col-0                    | 0                                |
| LpAFP                    | 0.11                             |
| LpIRI2                   | 0.08                             |
| LpIRI3                   | 0.13                             |
| A3                       | 0.15                             |
| 2A3                      | 0.18                             |
| (b)                      |                                   |
| Col-0                    | 0                                |
| mOrange                  | 0.025                            |
| mOrange-LpAFP            | 0.035                            |
| LpIRI2-mOrange           | 0.035                            |
| LpIRI3-mOrange           | 0.07                             |

Table 2 Mean fluorescence readings (wavelength emission and excitation of 562 nm and 548 nm, respectively) obtained using apoplast extracts (0.1 mg/mL total protein; triplicate samples) of mOrange-tagged LpIRIP lines compared with wild-type A. thaliana plants and plants expressing mOrange alone

| Transgenic line          | Relative fluorescence units (RFUs) |
|--------------------------|-----------------------------------|
| mOrange                  | 1037                              |
| mOrange-LpAFP            | 1100                              |
| LpIRI2-mOrange           | 12 834                            |
| LpIRI3-mOrange           | 13 474                            |

Values were normalized based on the level of fluorescence emitted in control, nontransgenic lines.
that observed with LpIRI3-expressing lines, the fact that recombinant LpIRI2 showed a reduced ability to restrict ice crystal growth compared with LpIRI3 (Figure 2) suggests that such differences may be due to varying affinities for ice crystal planes or less efficient ice crystal adsorption. Attempts to localize this isoform, as well as other IBPs, using fluorescently tagged proteins in planta did not allow us to unambiguously distinguish between the intracellular plasma membrane and the apoplast (Supplementary 1). Nevertheless, IRI and ice-shaping activity, as well as fluorescence, were present in apoplast extracts and guttation fluid of LpIRI2-expressing A. thaliana lines (Figures 5 and 7, and Table 2). Additionally, the level of freeze tolerance observed in LpIRI2 lines was superior to that seen for the intracellular LpAFP lines, in accordance with extracellular localization. Therefore, taken together, these observations support the hypothesis that LpIRI2 is a secreted protein.

Protein secretion using nonclassical pathways is a relatively unexplored area of research. Despite this, it has been estimated that 60% of proteins identified in the secretome of A. thaliana are leaderless secreted proteins (LSPs) (Regente et al., 2012). The mechanisms involved in the recognition of LSPs are not well known, and may not be conserved amongst or within secretion systems, suggesting that such mechanisms have evolved independently. Therefore, reliable prediction is not possible. Intriguingly, a nonclassical secretion system appears more common amongst protein families involved in environmental stress responses (Cheng et al., 2009; Gupta and Deswal, 2012; Kim et al., 2008). Thus, it is possible that non-Golgi secretion could provide flexible spatial localization, allowing proteins to take on dual function roles inside and outside of the cell. Perhaps as important, given the large number of proteins up-regulated during the freezing stress response, nonclassical secretion could provide an alternative, and potentially more efficient secretion for critical proteins under these circumstances, allowing other necessarily ER-linked proteins to monopolize the ‘traditional’ pathway.

Secretion through the ER-Golgi pathway is required for the post-translational modification of proteins. In this regard, a

Figure 5 Ice-binding phenotypes in apoplast extracts collected from transgenic A. thaliana plants bearing fluorescently tagged LpIRIP sequences. Shown are the ice crystals seen during ice recrystallization inhibition analysis following an 18-h annealing period at −4 °C (a) and the corresponding ice crystal morphologies (b). The apoplast extracts of transgenic lines expressing fluorescently tagged LpIRIP constructs were compared to control (Col-0) nontransgenic plants, at a total protein concentration of 0.1 mg/mL. Only one representative sample is shown for each LpIRIP-expressing line. Both assays were conducted in duplicate.

© 2016 The Authors. Plant Biotechnology Journal published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd., 15, 68-81
number of putative glycosylation sites have been identified in LpIRIP family members (Kuiper et al., 2001). Nevertheless, recombinantly produced LpAFP retains IRI and TH activities, indicating that such modifications are not necessary for proper ice-binding activity (Lauersen et al., 2011) and there has been no demonstration that LpIRIPs are post-translationally modified in planta. Further experiments regarding the localization of IBPs lacking classical secretion signals, as well as the possible nonclassical mechanisms of protein secretion, could prove useful in understanding the roles that IBPs play in plant freeze tolerance.

We believe that a lack of knowledge regarding the mechanisms underlying IBP-induced freezing tolerance has hindered the development of freeze-tolerant crops. In the past, a great deal of effort has been invested into the transfer of fish and insect AFPS to plants (e.g. Hightower et al., 1991; Holmberg et al., 2001; Huang et al., 2002; Kenward et al., 1999). These transgenes were considered attractive candidates given the ability of these proteins to depress the freezing point by several degrees. However, the expression of moderately TH-active fish AFPS in plants has not yielded favourable results, likely due to the catastrophic needle-like crystals that are formed once the freezing point is reached. These ice crystal burst patterns are due to

---

**Figure 6** Ice recrystallization inhibition (IRI) analysis of plants expressing fluorescently tagged LpIRIPs. Crude cell extracts were annealed at −4 °C for 18 h. Samples were assayed at a total protein concentration of 0.1 mg/mL. Only one representative sample is shown for each transgenic line. Assays were performed in triplicate.

**Figure 7** Ice recrystallization inhibition analysis of guttation fluid collected from transgenic LpIRIP-expressing A. thaliana plants. Guttation fluid collected from LpAFP-, LpIRI3- and LpIRI2-expressing lines was used for a splat assay. Samples were held at −4 °C for 24 h and compared to nontransgenic control plants (Col-0). A total protein concentration of 0.1 mg/mL was used for all experiments. Only one representative sample is shown for each LpIRIP-expressing line. Assays were conducted in triplicate.
that have evolved in species where freezing of the interstitial fluid strategy would logically appear to be more promising than AFPs the use of IBPs that have evolved from a freezing tolerant survival given the inevitability that most overwintering plants will freeze, IBPs, they have high TH activity. This could allow intracellular midal crystals, similar to those produced in the presence of plant AFPs. Plants expressing LpAFP (a), LpIRI3 (b) and LpIRI2 (c) constructs following incubation at 4 °C or freezing to −6 °C. Transgenic plants were compared to nontransgenic A. thaliana plants (Col-0). Ion leakage is represented as the proportion of ions leaked following treatment in relation to the total number of ions in the leaf sample. All experiments were performed in triplicate (n = 12). Error bars represent standard errors of the mean and asterisks denote a significant reduction in ion leakage compared with controls (*P < 0.05, **P < 0.005, ***P < 0.0005; two-tailed t-tests).

Figure 8

| Line          | Col-0 | LpAFP (a) | LpAFPb | LpAFPC | LpAFPD |
|---------------|-------|-----------|--------|--------|--------|
| Ions leaked (%) |      |           |        |        |        |
| 4 °C          | 90    | 95        | 95     | 95     | 95     |
| −6 °C         | 85    | 90        | 90     | 90     | 90     |

| Line          | Col-0 | LpIRI3a   | LpIRI3b | LpIRI3c | LpIRI3d |
|---------------|-------|-----------|---------|---------|---------|
| Ions leaked (%) |      |           |         |         |         |
| 4 °C          | 90    | 95        | 95      | 95      | 95      |
| −6 °C         | 85    | 90        | 90      | 90      | 90      |

| Line          | Col-0 | LpIRI2a   | LpIRI2b | LpIRI2c | LpIRI2d |
|---------------|-------|-----------|---------|---------|---------|
| Ions leaked (%) |      |           |         |         |         |
| 4 °C          | 90    | 95        | 95      | 95      | 95      |
| −6 °C         | 85    | 90        | 90      | 90      | 90      |

adsorption of AFPs to the primary prism plane exclusively, resulting in growth from the c-axis (Fletcher et al., 2001). Although insect AFPs direct the formation of hexagonal bipyramidal crystals, similar to those produced in the presence of plant IBPs, they have high TH activity. This could allow intracellular freezing at the same time or earlier than extracellular freezing, which would not be compatible with crop survival. We suggest that plant IBPs with their low TH activity, but relatively high IRI activity and ‘gentle’ burst morphologies (Middleton et al., 2012), should prove more efficacious for such applications. Certainly, given the inevitability that most overwintering plants will freeze, the use of IBPs that have evolved from a freezing tolerant survival strategy would logically appear to be more promising than AFPs that have evolved in species where freezing of the interstitial fluid is lethal. Here, we have shown that the expression of LpIRIPs in A. thaliana produced a freeze-tolerant phenotype that was enhanced in the presence of more than one isofrom. These results strongly suggest that the expression of multiple LpIRIP isoforms in a cold-adapted but freeze-susceptible crop may allow for even more substantial freezing tolerance capabilities than the striking −2 °C seen here.

Experimental procedures

Bioinformatics analysis

The translated sequences corresponding to the open reading frames (ORFs) of LpIRI1 (GenBank accession no. EU680848), LpIRI2 (EU680849), LpIRI3 (EU680850), LpIRI4 (EU680851) and
compared with Col-0 plants (*A. thaliana plants (Col-0). Error bars represent the standard errors of the mean and asterisks denote lines with significantly enhanced freezing survival (c) expressing LpIRI2. Survival was calculated for A. thaliana plants expressing multiple LpIRIP isoforms. Shown are the survival rates of LpAFP- (a) LpIRI3- (b) and LpIRI2- (c) expressing A. thaliana following recovery from temperatures between 0 and –8 °C. Transgenic plants were compared to nontransgenic A. thaliana plants (Col-0). Error bars represent the standard errors of the mean and asterisks denote lines with significantly enhanced freezing survival compared with Col-0 plants (*P < 0.05, **P < 0.005; two-tailed t-tests). Experiments were conducted using 100 seedlings for each independently generated transgenic line, in triplicate.

LpAFP (AJ277399) were aligned using ClustalW2 multiple sequence alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Predictions regarding the IRI-domain and putative ice-binding residues for LpIRIP sequences were made based on alignment with LpAFP, for which the IBF has been well characterized (Middleton et al., 2009). SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) was used to predict sequences encoding putative signal peptides. The Phyre 2.0 algorithm (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) was used to predict secondary protein structure.

Cloning and protein purification of recombinant LpIRIPs

Sequences corresponding to the ORFs of LpIRI2 and LpIRI3 were synthesized by GeneArt<sup>TM</sup> (Invitrogen, Carlsbad, CA), and the stop codons were removed by PCR with the primers LpIRI2NdCIFW/LpIRI2nostopXhoIRV (Table 3a) for LpIRI2 and LpIRI3NdCIFW/LpIRI3nostopXhoIRV (Table 3a) for LpIRI3, in order to incorporate a 6-residue histidine tag to facilitate protein purification. Amplification was performed using Platinum<sup>®</sup> Pfu Taq DNA polymerase (Invitrogen, Carlsbad, CA) using the following program: 2 min at 94 °C followed by 35 cycles of 30 sec at 94 °C, 30 s at 54 °C and 1 min 50 s at 72 °C, with a final extension at 72 °C for 7 min. The amplified products were then ligated into pET24a(+) vectors (Novagen, Ettobicoke, ON, CA) and transformed into ArcticExpress<sup>™</sup> Escherichia coli cells (New England Biolabs Inc., Whitby, ON, CA) using chemical transformation, with each construct subsequently confirmed by sequencing (Plateforme de séquençage et de génotypage des genres; Québec City, QC, CA) after each cloning step.

Bacterial cultures were grown to an optical density (OD) of 0.8 (λ = 595) and induced for 48 h at 16 °C, using 0.5 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were lysed using a French Press (ThermoFisher Scientific, Nepean, ON, CA) and recombinant proteins purified from soluble lysates using a nickel-NTA agarose column (Qiagen, Toronto, ON, CA) as previously described (Lauersen et al., 2011). Purified proteins were dialysed against 50 mM Tris–HCl and 100 mM NaCl, pH 8.0, for 24 h and used immediately for ice-binding and protein assays or frozen at –20 °C until analysed.

Cloning of LpIRIP constructs for expression in A. thaliana

For expression in A. thaliana, the ORFs of LpAFP, LpIRI2 and LpIRI3 were PCR-amplified using LpAFPBglIIFW/LpAFPnIRV, LpIRI2BglIIFW/LpIRI2PmlIRV and LpIRI3BglIIFW/LpIRI3PmlIRV primers, respectively (Table 3a) with the following protocol: 95 °C
for 2 min followed by 35 cycles of 95 °C for 45 s, 50 °C for 45 s and 72 °C for 1 min, with a final extension of 7 min at 72 °C. All fragments were ligated into pCAMBIA1305.1 vectors (Cambia, Canberra, ACT, AU) under the control of a cauliflower mosaic virus (CaMV) 35S promoter and a nopaline synthase (NOS) terminator. The construct for the simultaneous expression of LpIRI3 and LpAFP (A3) was generated by PCR amplification of 35S:LpAFP:NOS with 35S8amHIFW/NOSTerHindIIIIRV primers (Table 3a), using the protocol described above, and inserting the amplified fragment into the pCAMBIA1305.1:LpIRI3 construct. Subsequently, 35S:LpIRI3:NOS was amplified using 35SSecoRFW/NOSTerSacIRV (Table 3a) and inserted into the A3 construct for expression of all three isoforms (2A3), again using the same program.

The gene sequence corresponding to mOrange-LpAFP was ligated into pCAMBIA1305.1 following PCR amplification using OFPBglIIFW/LpAFPPrmIRV primers (Table 3a) under the following thermocycler program: 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 1 min 30 s with a final extension of 72 °C for 10 min. LpIRI2 and LpIRI3 were amplified using LpIRI2NcoIFW/LpIRI2nostopBgIIIRV and LpIRI3NcoIFW/LpIRI3nostopBgIIIRV primers (Table 3a), respectively, using the PCR conditions described for recombinant LpIRIP constructs, and inserted upstream of the mOrange tag to avoid disruption of the N-terminal signal sequence. In contrast, by placing the mOrange tag upstream of LpAFP, we could ensure that this protein remained intracellular, serving as control for cytoplasmic localization. Gene sequences were again confirmed by sequencing.

Plant materials and growth conditions

Lolium perenne seeds (Pacific Seed diploid variety; Premier Specific Seeds, Surrey, BC, CA) used for transcript analysis were grown in potting soil and maintained in a growth chamber (Queen’s University, Kingston, ON, CA) on a 20-h/4-h light/dark cycle at 22 °C/18 °C with humidity and light regulated at 70% and 175 μmol/m²s, respectively. Prior to reverse transcription (RT) PCR analysis, plants were grown for 3 weeks prior to a 6-day acclimation period at 21 °C (no light; control) or a cold acclimation period at 4 °C (no light).

All transgenic expression experiments were conducted using wild-type A. thaliana (ecotype: Col-0). For crude cell extractions, apoplast extraction, collection of guttation fluid and electrolyte leakage assays, A. thaliana were sown in potting soil and grown in a growth chamber for 3 weeks under standard growth conditions with a 16-h/8-h light/dark cycle at 22 °C/20 °C, 70% relative humidity, and light ~150 μmol/m²s. Seeds used for survival assays were surface-sterilized using 70% (v/v) ethanol with 0.05% (v/v) Triton X-100 for 5 min, followed by a 95% (v/v) ethanol wash for 5 min, and plated on 0.5× Murashige and Skoog (MS) agar prior to transfer to standard growth conditions.

Prior to experimentation, plants were cold-acclimated (CA) at 4 °C, on a short-day cycle (6-h/18-h light/dark), with ~175 μmol/m²s light for 48 h. These conditions were imposed for two reasons: first, we sought to prevent LpIRIP misfolding, which occurs above 16 °C (Lauersen et al., 2011), and secondly, we wished to limit the time at 4 °C, because A. thaliana reaches...
optimal freezing tolerance after 1 week of low-temperature exposure (Uemura et al., 1995). Thus, we attempted to balance the accumulation period of well-folded LpIRIPs and at the same time reduce the possible confounding effect of up-regulating other cold-induced freezing tolerance host mechanisms.

Generation of A. thaliana transgenic lines
Tagged and untagged LpIRIP constructs designed for expression in A. thaliana were transformed into GV3101 Agrobacterium tumefaciens cells by electroporation. Agrobacterium-mediated transformation of A. thaliana was carried out using the floral dip method (as described in Middleton et al., 2014). Successfully transformed plants were selected on 0.5x MS medium plates containing hygromycin (50 µg/mL). Four independent, homozygous lines were generated for each construct.

Endogenous LpIRIP transcript analysis
RNA was collected from the leaf tissue of CA L. perenne grown under the conditions described above. Extractions were performed using the RNeasy Plant Mini Kit (Qiagen, Toronto, ON, CA) followed by cDNA synthesis using SuperscriptIII First-Strand Synthesis System (Invitrogen, Carlsbad, CA) according to the manufacturers’ specifications. RT-PCR was carried out using the following cycle conditions: 95 °C for 5 min, 94 °C for 30 s, 53 °C for 30 s and 72 °C for 1 min, for 45 cycles. LpIRI2 was amplified using LpIRI2F/SamDC primers and LpIRI3 was amplified using LpIRI3F/SamDC primer sets (Table 3b). A housekeeping gene, s-adenosylmethionine decarboxylase transcript (SamDC), was amplified with primers as previously described (Hong et al., 2008) using the following cycle conditions: 95 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s.

Ice-binding and protein assays
Ice-binding assays were performed using protocols that have been optimized for plant IBPs as described previously. IRI activity was assessed using splat assays and ice-shaping activity and TH activity assayed using a nanolitre osmometer (Middleton et al., 2014). Prior to analysis, crude cell lysates (Lauersen et al., 2011), apoplast extracts (Villers and Kwak, 2013) and guttation fluid (Madsen et al., 2016) were prepared from 4-week-old CA A. thaliana leaf tissue as described previously. Plant extracts were suspended in a native protein extraction buffer (10 mM Tris–HCl, 25 mM NaCl, pH 7.5). Recombinant proteins used for ice-binding assays were prepared as described above. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Nepean, ON, CA) following the manufacturer’s instructions, with all experiments carried out in triplicate.

Electrolyte leakage assay
Electrolyte leakage assays using 4-week-old CA A. thaliana plants were carried out using a modified protocol from Thalhammer et al. (2014). Two mature rosette leaves were cut and placed in glass tubes containing deionized water (100 µL). Treatments were conducted by placing tubes in a circulating water bath at 0 °C and lowering the temperature to −1 °C, over 30 min. Samples were then nucleated using a single ice chip, and the temperature was decreased (1 °C every 15 min) to −6 °C. Following treatment, experimental samples were removed from the circulating water bath and allowed to recover overnight at 4 °C in the dark. Control samples were left covered in the dark at 4 °C, while treated experimental samples were prepared and allowed to recover at 4 °C overnight. All cut leaf samples were then transferred to conical tubes (50 mL containing 20 mL of deionized water) and shaken for 18 h at 24 °C, 150 rpm. Initial (C0) and final conductivity (Cf) measurements were taken before and after autoclaving samples using a direct reading conductivity meter (Bach-Simpson Ltd., London, ON, CA) and presented as a percentage (100 C/C0) with 12 individual plants for each independent line, in triplicate. Significance was evaluated using two-tailed t-tests (P < 0.05).

A. thaliana freeze survival assay
Whole plant freeze survival assays were modified from Xin and Browse (1998). A. thaliana seedlings (~2000 per experiment) were grown for 4 weeks on MS agar plates rather than soil to reduce the presence of any ice-nucleating bacteria. Plates were then transferred to −1 °C (no light) for 2 h prior to nucleation using an ice chip in the centre of the plate and kept at this temperature for 12 h to ensure that the agar was frozen. The temperature was then lowered by 1 °C every 2 h, until the temperature reached −8 °C. One plate for each line was removed at temperatures indicated in the Results (between −5 and −8 °C) and allowed to recover at 4 °C (no light) for 24 h, prior to transfer to standard growth conditions for 14 days, when per cent survival and LT50 were calculated. Assays were performed using 100 seedlings per independent line, in triplicate, with significant differences evaluated using a two-tailed t-test (P < 0.05).

Fluorescence readings
The level of mORANGE-tagged protein present in the apoplast extracts of transgenic A. thaliana plants was determined using the SpectraMax Gemini XS microplate reader ( Molecular Devices, Sunnyvale, CA). Emission and excitation wavelengths of 562 nm and 548 nm, respectively, were used based on the specifications of the mORANGE protein product. Readings were taken at a total protein concentration of 0.1 mg/mL.

Confocal microscopy
Two-week-old seedlings grown on 0.5x MS agar plates were cold-acclimated for 48 h at 4 °C (6 h light) prior to visualization. A laser scanning microscope (LSM710; Ziess, Oberkochen, BW, DE) was used to visualize mOrange-tagged proteins (~543 nm). Images were obtained using ZEN 2009 software.

Acknowledgements
We acknowledge Dr. P. Davies for use of his nanolitre osmometer and Dr. Moez Hanin for generously donating the mOrange-LpAFP construct. We also thank Dr. K. Lauersen and Lena Dolman for their efforts in preliminary studies. This work was funded by the NSERC Discovery grant to VKW.

References
Antikainen, M. and Griffith, M. (1997) Antifreeze protein accumulation in freezing-tolerant cereals. Physiol. Plant. 99, 423–432.
Bar-Dolev, M., Celik, Y., Wettlaufer, J.S., Davies, P.L. and Braslavsky, I. (2012) New insights into ice growth and melting modifications by antifreeze proteins. J. R. Soc. Interface, 9, 3249–3259.
Beirão, J., Zilli, L., Villela, S., Cabrita, E., Schiavone, R. and Herráez, M.P. (2011) Improving sperm cryopreservation with antifreeze proteins: effect on gilthead seabream (Sparus aurata) plasma membrane lipids. Biol. Reprod. 86, 1–9.
Burcham, T.S., Knauf, M.J., Osuga, D.T., Feehey, R.E. and Yeh, Y. (1984) Antifreeze glycoproteins influence of polymer length and ice crystal habit on activity. Biopolymers, 23, 1379–1395.

Cheng, F., Zamski, E., Guo, W.W., Pharr, D.M. and Williamson, J.D. (2009) Salicylic acid stimulates secretion of the normally symptomatic enzyme mannosyl dehydrogenase (MTD): a possible defense against mannosyl-secreting fungal pathogens. Planta, 230, 1093–1108.

Davies, P.L. and Hew, C.L. (2013) Biochemistry of fish antifreeze proteins. FASEB J. 4, 2460–2468.

Duman, J.G. (1994) Purification and characterization of a thermal hysteresis protein from a plant, the bittersweet nightshade Solanum dulcamara. Biochem. Biophys. Acta, 1206, 129–135.

Duman, J.G. (2001) Antifreeze and ice nucleator proteins in terrestrial arthropods. Annu. Rev. Physiol. 63, 327–357.

Fei, Y.B., Sun, L.H., Huang, T., Shu, N.H., Gao, S.Q. and Jian, L.C. (1994) Isolation and identification of antifreeze protein with high activity in Amomoptantus mongolicus (in Chinese with English Abstract). Acta Bot. Sin. 36, 649–650.

Fletcher, G.L., Hew, C.L. and Davies, P.L. (2001) Antifreeze proteins of teleost fishes. Annu. Rev. Physiol. 63, 359–390.

Gilbert, J.A., Hill, P.J., Dodd, C.E. and Laybourn-Parry, J. (2004) Demonstration of antifreeze protein activity in Antarctic lake bacteria. Microbiology, 150, 171–180.

Griffith, M., Ala, P., Yang, D.S., Hon, W.C. and Moffatt, B.A. (1992) Antifreeze protein produced endogenously in winter rye/leaves. Plant Physiol. 100, 593–596.

Huang, T. and Duman, J.G. (2002) Cloning and characterization of a novel antifreeze protein from the perennial grass, winter bittersweet nightshade, Solanum dulcamara. BMC Plant Biol. 2, 254–258.

Hon, W.C., Griffith, M., Chong, P. and Yang, D. (1994) Extraction and isolation of antifreeze proteins from winter rye (Secale cereale L.) leaves. Plant Physiol. 104, 971–980.

Hon, W.C., Griffith, M., Mlynarz, A., Kvik, Y.C. and Yang, D.S. (1995) Antifreeze proteins in winter rye are similar to pathogenesis-related proteins. Plant Physiol. 109, 879–889.

Hong, S.Y., Seo, P.J., Yang, M., Xiang, F. and Park, C. (2008) Exploring valid reference genes for gene expression studies in Brachypodium distachyon. BMC Plant Biol. 8, 492–4935.

Huang, T. and Duman, J.G. (2002) Expression of an insect (Dendroides canadensis) antifreeze protein in Arabidopsis thaliana results in a decrease in plant freezing temperature. Arch. Biochem. Biophys. 390, 249–253.

Huang, T. and Duman, J.G. (2002) Expression of antifreeze proteins in transgenic plants. Cryobiology, 45, 175–186.

Huang, T., Nicodemus, J., Zarka, D.G., Thomashow, M.F., Wisniewski, M. and Duman, J.G. (2002) Expression of an insect (Dendroides canadensis) antifreeze protein in Arabidopsis thaliana results in a decrease in plant freezing temperature. Plant Physiol. 129, 709–717.

Kajava, A. and Lindow, S.E. (1993) A model of the three-dimensional structure of ice nucleation proteins. J. Mol. Biol. 232, 709–717.

Kenward, K.D., Brandle, J., McPherson, J. and Davies, P.L. (1999) Type II fish antifreeze protein accumulation in transgenic tobacco does not confer frost tolerance. Transgenic Res. 8, 105–117.

Kim, H.J., Kato, N., Kim, S. and Trittle, B. (2008) CuZn superoxide dismutase in developing cotton fibers: evidence for an extracellular form. Planta, 228, 281–292.

Kuiper, M.J., Davies, P.L. and Walker, V.K. (2001) A theoretical model of a plant antifreeze protein from Lollum perene. Biophys. J. 81, 3560–3565.

Kumble, K.D., Demmer, J., Fish, S.A., Hall, C., Corrales, S., DeAth, A., Elton, C., Prestidge, R., Luxmanan, S., Marshall, C.J. and Wharton, D.A. (2008) Characterization of a family of ice-active proteins from the Ryegrass, Lollum perene. Cryobiology, 57, 263–268.

Lauersen, K.J., Brown, A., Middleton, A., Davies, P.L. and Walker, V.K. (2011) Expression and characterization of an antifreeze protein from the perennial ryegrass, Lollum perene. Cryobiology, 62, 194–201.

Madsen, S.R., Nour-Eldin, H.H. and Halkier, B.A. (2016) Collection of Apoplastic Fluids from Arabidopsis thaliana leaves. Methods Mol. Biol. 1405, 35–42.

Marentez, E., Griffith, M., Mlynarz, A. and Brush, R.A. (1993) Proteins accumulate in the apoplast of winter rye leaves during cold-acclimation. Physiol. Plant. 87, 499–507.

Middleton, A.J., Brown, A.M., Davies, P.L. and Walker, V.K. (2009) Identification of the ice-binding face of a plant antifreeze protein. FEMS Lett. 583, 815–819.

Middleton, A.J., Marshall, C.B., Faucher, F., Bar-Dolev, M., Braslavsky, I., Campbell, R.L., Walker, V.K. and Davies, P.L. (2012) Antifreeze protein freezing-tolerant grass has a beta-roll fold with an irregularly structured ice-binding site. J. Mol. Biol. 416, 713–724.

Nishiyama, Y., Sato, R., Takamichi, M., Miura, A. and Tsudo, S. (2005) Co-operative effect of the isoforms of type III antifreeze protein expressed in Notched-fin eelpot, Zoarces elongatus. Kner. FEMS J. 272, 482–492.

Pertaya, N., Marshall, C.B., Celik, Y., Davies, P.L. and Braslavsky, I. (2008) Direct visualization of spruce budworm antifreeze protein interacting with ice crystals: basal plane affinity confers hyperactivity. Biophys. J. 95, 333–341.

Pudney, P.D., Buckley, S.L., Sidebottom, C.M., Twigg, S.N., Sevilla, M.P., Holt, C.B., Roper, D. et al. (2003) The physico-chemical characterization of a boiling stable antifreeze protein from a perennial grass (Lollum perene). Arch. Biochem. Biophys. 410, 238–245.

Raymond, J.A. and DeVries, A.L. (1977) Adsorption inhibition as a mechanism of freezing resistance in polar fishes. Proc. Natl Acad. Sci. USA, 74, 2589–2593.

Regente, M., Pinedo, M., Elizalde, M. and de la Canal, L. (2012) Apoplastic exosome-like vesicles: a new way of protein secretion in plants? Plant Signal Behav. 7, 544–546.

Rubinsky, B., Arav, A. and Fletcher, G.L. (1991) Hypothetical protection: a fundamental property of “antifreeze” proteins. Biochem. Biophys. Res. Commun. 180, 566–571.

Sandve, S., Rudi, H., Asp, T. and Rogr, O. (2008) Tracking the evolution of a cold stress associated gene family in cold tolerant grasses. BMC Evol. Biol. 8, 1–15.

Sandve, S.R., Kosmala, A., Rudi, H., Fjellheim, S., Rapacz, M., Yamada, T. and Rogr, O.A. (2011) Molecular mechanisms underlying frost tolerance in perennial grasses adapted to cold climates. Plant Sci. 180, 69–77.

Sidebottom, C., Buckley, S., Pudney, P., Twigg, S., Jarman, C., Holt, C., Telford, J. et al. (2000) Phytochemistry: heat-stable antifreeze protein from grass. Nature, 406, 256–256.

Siminovitch, D., Singh, J. and De La Rouche, I.A. (1978) Freezing behavior of free protoplasts of winter rye. Cryobiology, 15, 205–213.

Simpson, D., Smallwood, M., Twigg, S., Douct, C.J., Ross, J. and Bowles, D.J. (2005) Purification and characterization of a novel antifreeze protein from Forsythia suspensa (L.). Cryobiology, 51, 230–234.

Smallwood, M., Worrall, D., Byass, L., Elias, L., Ashford, D., Douct, C.J., Holt, C. et al. (1999) Isolation and characterization of a novel antifreeze protein from carrot (Daucus carota). Biochem. J. 340, 385–391.

Thalhammer, A., Hincha, D.K. and Zuber, E. (2014) Measuring freezing tolerance: electrolyte leakage and chlorophyll fluorescence assays. Methods Mol. Biol. 1166, 15–24.

Thomas, H. and James, A.R. (1993) Freezing tolerance and solute changes in contrasting genotypes of Lolium perene L. acclimated to cold and drought. Ann. Bot. 72, 249–254.

Thomashow, M.F. (1998) Role of cold-responsive genes in plant freezing tolerance. Plant Physiol. 118, 1–8.
Tomalty, H. and Walker, V.K. (2014) Perturbation of ice nucleation activity by an antifreeze protein. Biochem. Biophys. Res. Commun. 452, 636–641.

Tomczak, M. M., Hincha, D. K., Estrada, S. D., Feeley, R. E. and Crowe, J. H. (2001) Antifreeze proteins differentially affect model membranes during freezing. Biochem. Biophys. Acta, 155, 255–263.

Uemura, M., Joseph, R. A. and Steponkus, P. L. (1995) Cold acclimation of Arabidopsis thaliana. Plant Physiol. 109, 15–30.

Villers, F. and Kwak, J. M. (2013) Rapid apoplastic pH measurement in Arabidopsis leaves using a fluorescent dye. Plant Signal. Behav. 8, e22587.

Wallis, J. G., Wang, H. and Guerra, D. J. (1997) Expression of a synthetic antifreeze protein in potato reduced electrolyte leakage release at freezing temperatures. Plant Mol. Biol. 35, 323–330.

Xin, Z. and Browse, J. (1998) eskimo1 mutants of Arabidopsis are constitutively freezing-tolerant. PNAS, 95, 7799–7804.

Xu, H., Griffith, M., Patten, C. L. and Glick, B. R. (1998) Isolation and characterization of an antifreeze protein with ice-nucleation activity from the plant growth promoting rhizobacterium Pseudomonas putida GR12-2. Can. J. Microbiol. 44, 64–73.

Zhang, C., Fei, S. Z., Arora, R. and Hannapel, D. J. (2010) Ice recrystallization inhibition proteins of perennial ryegrass enhance freezing tolerance. Planta, 232, 155–164.

Supporting information

Additional Supporting information may be found online in the supporting information tab for this article:

Figure S1 Expression of fluorescently tagged LpIRIP constructs in the roots of transgenic A. thaliana plants. Roots were visualized using a confocal microscope. Experiment was performed in duplicate.