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Perturbation of bacterial ice nucleation activity by a grass antifreeze protein

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ABSTRACT

Certain plant-associating bacteria produce ice nucleation proteins (INPs) which allow the crystallization of water at high subzero temperatures. Many of these microbes are considered plant pathogens since the formed ice can damage tissues, allowing access to nutrients. Intriguingly, certain plants that host these bacteria synthesize antifreeze proteins (AFPs). Once freezing has occurred, plant AFPs likely function to inhibit the growth of large damaging ice crystals. However, we postulated that such AFPs might also serve as defensive mechanisms against bacterial-mediated ice nucleation. Recombinant AFP derived from the perennial ryegrass *Lolium perenne* (*Lp*AFP) was combined with INP preparations originating from the grass epiphyte, *Pseudomonas syringae*. The presence of INPs had no effect on AFP activity, including thermal hysteresis and ice recrystallization inhibition. Strikingly, the ice nucleation point of the INP was depressed up to 1.9 °C in the presence of *Lp*AFP, but a recombinant fish AFP did not lower the INP-imposed freezing point. Assays with mutant *Lp*AFPs and the visualization of bacterially-displayed fluorescent plant AFP suggest that INP and *Lp*AFP can interact. Thus, we postulate that in addition to controlling ice growth, plant AFPs may also function as a defensive strategy against the damaging effects of ice-nucleating bacteria.

1. Introduction

Ice-binding proteins function as part of a survival strategy for some organisms that cannot avoid exposure to subzero temperatures. These proteins include antifreeze proteins (AFPs) and ice nucleation proteins (INPs), which manipulate the growth of ice or the crystallization temperature. First discovered in insects [1], then in polar fish [2], AFPs adsorb to embryonic ice crystals resulting in a depression of the freezing point relative to the melting point [3]. The difference between the melting and freezing temperature is measured as the thermal hysteresis (TH) activity. In plants, AFPs have been isolated from several species including the perennial ryegrass, *Lolium perenne* [4,5]. Generally, plant AFPs are characterized by lower TH activity compared to the AFPs found in some insects and polar fish. Since certain plants cannot avoid freezing, the primary function of a plant AFP is to inhibit the growth of large, damaging ice crystals, with little impact on freezing point depression [5]. Because of this characteristic, plant AFPs are also referred to as ice recrystallization (IR) inhibitors.

INPs operate in a seemingly opposite manner compared to AFPs, functioning as heterogeneous nucleators that catalyze ice crystallization at high subzero temperatures. Although several different organisms have been reported to have ice nucleation activity (INA), only those INPs produced by bacteria have been well characterized. INPs have been isolated from approximately ten different bacterial species belonging to at least three different genera: *Pseudomonas*, *Erwinia*, and *Xanthomonas* [6], with the encoding DNA sequences almost certainly exchanged by horizontal transfer between species [7]. INPs form aggregates on the outer membrane where they function as a template for ice formation [8].

INP-producing, plant-associating bacteria are frequently viewed as plant pathogens, but this is not always the case. Certainly it is recognized that several epiphytic bacteria produce INPs as a way of initiating wounding to leaves and stems, permitting access to a rich pool of nutrients [9]. Some of these bacteria are also known to invade the plant during favorable conditions, gaining access to the apoplast through openings on the plant's surface [10]. In surveys of *L. perenne* leaves, 40% of the bacterial community was represented by *Pseudomonas fluorescens*, *Pseudomonas* spp., *Erwinia herbicola*, and *Xanthomonas campestris*, all associated with INP production [11].

As indicated, *L. perenne* AFP (*Lp*AFP) is postulated to offer host protection by inhibiting IR once freezing has occurred [5]. Although

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this is undoubtedly true, we wondered if there was also an interaction between the bacterial INPs and the AFPs in the plant extracellular fluids. In this regard, the evolutionary origin of *Lp*AFP is unknown; some of these plant AFP sequences appear to be related to defensive agents such as pathogenesis-related proteins [12,13]. Could *Lp*AFP also play a defensive role, offering protection not only from IR, but also against the INA of potentially pathogenic bacteria? Previously, it has been suggested that insect AFPs and INPs could interact [14,15], but to our knowledge, quantified studies and detailed characterization of any interactions have not been done. It is also important to test the interaction of INPs and AFPs derived from other species to determine if any such interaction is specific to plant AFPs. Such analysis, we hope, will also contribute to our structural and functional understanding of these two distinct ice-associating proteins.

2. Materials and methods

2.1. Protein and sample preparation

Recombinant AFPs including *Lp*AFP (GenBank: AJ277399), two mutated versions of *Lp*AFP (N72Y and T43Y), *Lp*AFP tagged with green fluorescent protein (*Lp*AFP-GFP), and fish type III AFP derived from the ocean pout, *Macrozoarces americanus* were purified as previously described [16–19].

P. syringae INP preparations were purchased from Ward's Natural Science (USA) and used at concentrations ranging from 50 to 5 μg/ml. *P. syringae* B728a [20] and *Pseudomonas borealis* DL7 [21] were cultured for 24–48 h at 22 °C in 10% tryptic soy broth (TSB) and subsequently cold conditioned for two days at 4 °C before used as an additional source of INPs [22]. Cytochrome C was used as a control to distinguish any protein-mediated concentration effects.

2.2. Ice nucleation assays

Ice nucleation activity (INA) was assayed using a procedure modified from a standard technique [23]. Briefly, freezing points were obtained by pipetting 20 replicate samples (2 μl) on a polarized film, which was subsequently placed over an insulated chamber containing 50% ethylene glycol. While lowering the chamber temperature (−1 to −12 °C at 0.2 °C/min), images of the polarized film as well as the thermistor output were automatically recorded every 60 s. The temperature at which 90% of the samples froze (T_{90}) was considered the nucleation point, while samples with freezing points below −9 °C were not considered to have significant INA. Using Vali's [23] equation, the cumulative number of ice nuclei per ml in each sample ($K(T)$) was calculated as:

$$K(T) = -\ln(N(T)/N_0) * V - 1$$

with $N(T)$ representing the number of unfrozen drops at temperature T, N_0 representing the total drop number, and V representing the drop volume. INA (20 replicate samples) was determined at least three times with different protein preparations at all reported concentrations.

2.3. Antifreeze activity assays

IR inhibition was assayed using capillary assays [24] and a modified version of the splat assay [25], exactly as described [26]. A Clifton nanolitre osmometer was used to determine the TH of the AFPs [27], as well as to visualize ice crystal morphology [26]. All IR inhibition and TH assays were performed three or more times.

2.4. Fluorescence microscopy

Purified *Lp*AFP-GFP (to a final concentration of 0.5 mg/ml) was added to 1 ml aliquots of the cold-acclimated *P. syringae* or *P. borealis* cultures and allowed to incubate for 30 min at 4 °C. Samples (5 μl) were placed on clean microscope slides and visualized using a cold stage (Physiotemp Inc.) set at 4 °C on an inverted Zeiss Axiovert 200 M microscope under fluorescent light conditions (543 nm).

3. Results

3.1. Impact of INPs on AFP activity

Samples of recombinant AFPs were mixed with INP preparations and assayed for IR inhibition activity (Fig. 1A) and for changes in the morphology of individual ice crystals at their equilibrium temperature (Fig. 1B). Ice crystals formed in the presence of INPs

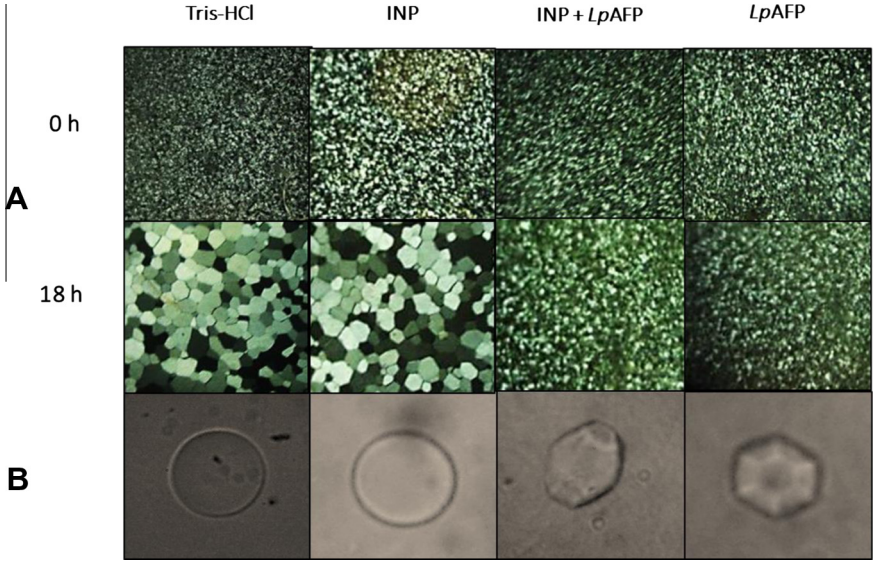

Fig. 1. Representative ice crystals from an IR inhibition splat assay (A) and typical ice crystal morphologies (B) in the presence of *P. syringae* ice nucleation protein (INP; 0.05 mg/ml) and *L. perenne* AFP (*Lp*AFP; 1 mg/ml). Both assays were performed in triplicate.

alone at any of the tested concentrations were disk-like, reflecting uninhibited ice growth on all six sides of the embryonic crystal (Fig. 1B). Ice crystals grown in solutions containing any of the tested AFPs (type III AFP, wild type *Lp*AFP, or *Lp*AFPs bearing mutations on the non-ice binding face) resulted in morphologies that were clearly distinct. Ice crystals were either bipyramidal (type III AFP) or hexagonal (*Lp*AFPs) in shape. The addition of INPs to any of these AFPs at any of the tested concentrations resulted in no visible disruption to the AFP-dependent appearance of the ice crystals (Fig. 1B).

In the absence of INPs, the mean TH values of the type III and *Lp*AFP preparations (all at 1 mg/ml) were 0.62 ± 0.1 and 0.20 ± 0.02 °C, respectively. After the addition of the INP preparations, the TH values did not change significantly (two-tailed, unpaired *t*-test, $P > 0.05$) with mean values of 0.59 ± 0.01 and 0.21 ± 0.02 °C for the fish and plant proteins, respectively. Predictably, both AFPs inhibited IR in either the capillary or splat assays, with ice crystals remaining small at annealing temperatures of −4 °C (Fig. 1A). In the absence of AFPs (buffer alone or control protein), these conditions resulted in large ice crystals. Similar to the TH assays, IR assays in the presence of INPs and any of the AFP samples showed that effective IR inhibition was maintained.

3.2. Impact of AFPs on INP activity

Initially, the impact of AFPs on INP activity was assessed using a concentration matrix with *P. syringae* preparations ranging from 50 to 0.5 μg/ml and AFP concentrations ranging from 0.05 to 3 mg/ml. Following that survey, a more thorough investigation was carried out using two different concentrations of type III AFP and *Lp*AFP (1 and 2 mg/ml) and two INP levels (50 and 5 μg/ml).

In the absence of AFPs, the mean INP-mediated freezing point was −3.08 and −3.82 °C (at 50 and 5 μg/ml, respectively). When type III AFP was added to the INP preparation, there was no significant depression of the freezing temperature at any of the tested concentrations compared to the non-AFP controls (two tailed, unpaired *t*-test; $P > 0.05$; Table 1). Fig. 2A represents typical results, showing the effect of type III AFP on the cumulative number of ice nuclei per ml of an INP preparation (at 50 μg/ml). At one concentration combination (2 mg/ml type III AFP and 5 μg/ml INP), however, there appeared to be a minor increase ($P < 0.05$) in the freezing temperature by an average of 0.33 °C.

In contrast to the results for type III AFP, the addition of *Lp*AFP (either 1.0 or 2.0 mg/ml) significantly depressed the freezing point of the INP preparations compared to non-AFP controls (two tailed, unpaired *t*-test; $P < 0.05$); *Lp*AFP depressed the freezing point of the INP up to 1.9 °C (Table 1). Graphically, the effects of *Lp*AFP on the cumulative number of ice nuclei are typified by Fig. 2B, which demonstrates the depression of the freezing point with the addition of *Lp*AFP (1 mg/ml) when added to the INP (50 μg/ml). The addition of the *Lp*AFP mutant T43Y (1 mg/ml) also significantly depressed the freezing point of tested INPs (Fig. 2C; two tailed, unpaired *t*-test, $P < 0.05$), but the *Lp*AFP mutant N72Y did not (Fig. 2D). The addition of *Lp*AFP-GFP also resulted in a significant depression of the INP freezing point, but the decrease was more modest than with *Lp*AFP, showing an average depression of 0.63 °C (Table 1).

To ensure the changes in the freezing temperatures were not due to protein colligative effects, cytochrome C was used as a control in all experiments. Cytochrome C did not alter the freezing temperature of INP preparations except at very high concentrations (>3 mg/ml). At these high concentrations, cytochrome C significantly altered the freezing point, consistent with the expected concentration dependent effect.

Table 1
Mean differences in freezing temperatures for *P. syringae* INP preparations in the presence or absence of recombinant AFPs.

Protein addition		[INP][a,b]	
		0.05 mg/ml	0.005 mg/ml
Type III AFP	2 mg/ml	+0.4 °C ± 0.2	+0.33 °C ± 0.3*
	1 mg/ml	+0.13 °C ± 0.4	+0.33 °C ± 0.3
*Lp*AFP	2 mg/ml	−1.9 °C ± 0.42*	−1.31 °C ± 0.29*
	1 mg/ml	−1.02 °C ± 0.31*	−0.9 °C ± 0.29*
*Lp*AFP-GFP	2 mg/ml	−0.39 °C ± 0.39	−0.86 °C ± 0.29*
	1 mg/ml	−0.63 °C ± 0.41*	−0.82 °C ± 0.17*
Cytochrome C	2 mg/ml	+0.21 °C ± 0.43	−0.08 °C ± 0.8
	1 mg/ml	+0.43 °C ± 0.34	−0.04 °C ± 0.27

[a] The standard deviation for each value is also shown. Each experiment was repeated three or more times.
[b] The mean freezing point in the presence of INPs alone was −3.08 °C ± 0.78 (0.05 mg/ml) and −3.82 °C ± 0.78 (0.005 mg/ml). These values were subtracted from the observed values to give the relative differences reported above.
* Values displayed with an asterisks (*) denotes samples that were significantly different from cytochrome C controls.

3.3. Visualization of the interaction between INPs and LpAFP

After cold conditioning *P. syringae* and *P. borealis* cultures for two days at 4 °C, ice nucleation assays, averaging −2.8 and −2.9 °C respectively, ensured that ice nucleating activity was present. Aliquots of these bacteria incubated with *Lp*AFP-GFP resulted in the appearance of concentrated green fluorescence at the poles of some cells in several samples of both *Pseudomonas* species (Fig. 3); however, it must be noted that these results were not always consistent. In this regard, it must be recalled that not all cells in *Pseudomonas* sp. cultures synthesize INPs, possibly explaining why fluorescence was not observed on every cell and in every slide [8].

4. Discussion

AFPs are thought to facilitate the freezing survival of perennials by inhibiting the growth of large ice crystals in the apoplast. However, we wondered if they also might serve to counter the damaging effects of INP-producing bacteria by depressing the freezing temperature. Such freezing point depression would restrict the initial ice crystal size and thus would presumably lead to reduced cell damage. Our results strongly support this hypothesis.

As far as we are aware, this hypothesis has not been previously investigated. However, others have examined the interaction of animal AFPs and ice nucleators, with sometimes conflicting results. For example, fish antifreeze glycoproteins (AFGPs) as well as insect AFPs were reported to inhibit bacterial INA [28,29] and fish type I and III AFPs depressed the nucleation temperature of AgI, an effective abiotic ice nucleator [30]. In contrast, type I and III AFP have been reported to actually enhance ice nucleation [31,32]. While the concept of an AFP, which lowers the freezing temperature, interacting with INPs to essentially promote ice nucleation in these studies would be seemingly contradictory, Holt [32] suggested that type III AFP could increase the freezing point of *P. syringae* by binding adjacent nucleators to form a larger surface area for ice nucleation. In this regard, it should be noted that we observed no AFP-associated enhancement of INA, with the single marginal exception involving the highest type III AFP concentration paired with the lowest INP concentration (Table 1). Other studies serve to further complicate the literature. For example, a bacterial AFP from *Pseudomonas putida* was shown to display a moderate level of ice nucleation activity [33]. Similarly, under certain circumstances including at high concentrations ($\geqslant$8 mg/ml), type I AFP acted as a nucleator [31]. It should be noted that fish are freeze intolerant

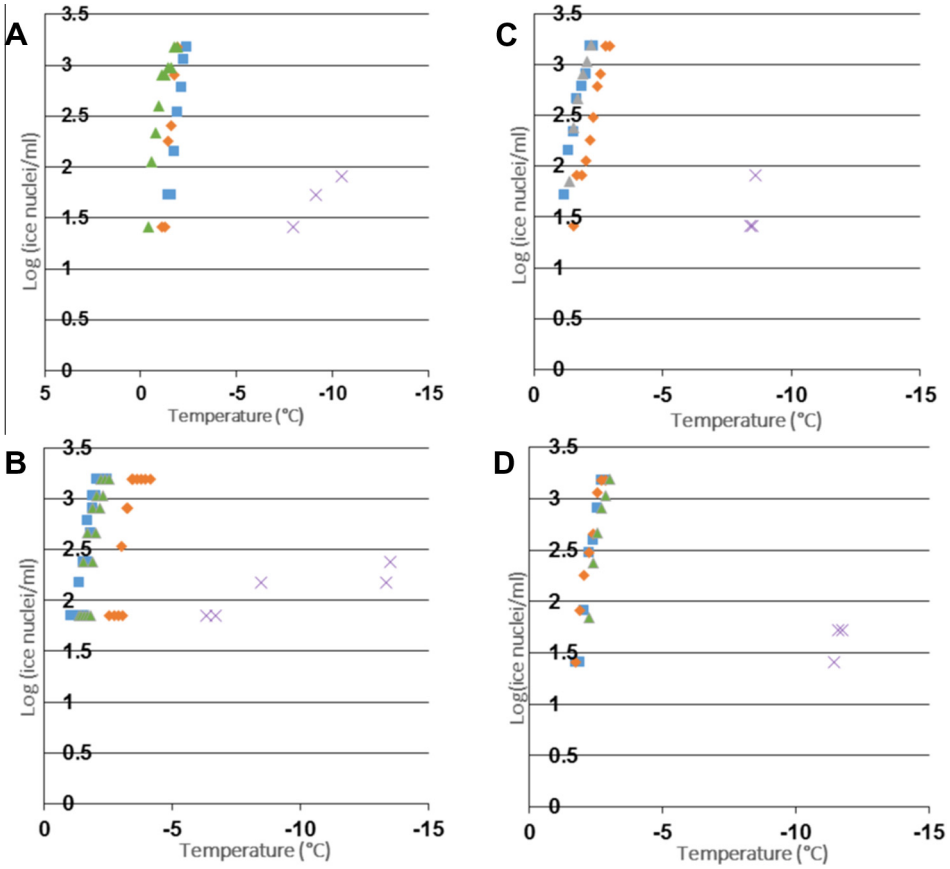

Fig. 2. Representative graphs for ice nucleation activity shown as the logarithm of the cumulative number of ice nuclei per ml with (A) type III (1 mg/ml), (B) *Lp*AFP (1 mg/ml), (C) *Lp*AFP T43Y (1 mg/ml) and (D) *Lp*AFP N72Y (1 mg/ml). Samples include an INP preparation from *P. syringae* (blue squares), INPs combined with an either type III AFP (A) or *Lp*AFP (B–D) (orange diamonds), INPs combined with cytochrome C (green triangles), and either type III AFP or *Lp*AFP alone (purple X). All ice nucleation assays were performed in triplicate.

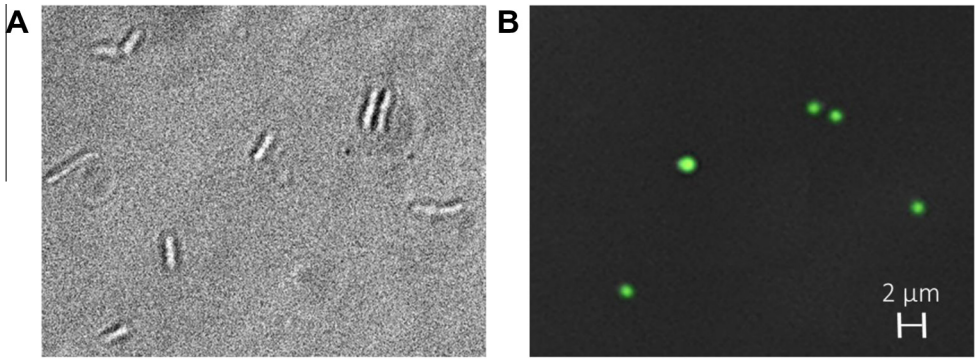

Fig. 3. Visualization of cold-acclimated *Pseudomonas borealis* DL7 cells incubated with GFP labeled *Lp*AFP. Cells viewed under normal/bright light conditions are shown in (A) and compared to those viewed under fluorescence (543 nm) in (B). Magnifications are shown by the bars. Note that not all bacteria show fluorescence.

organisms; if they freeze, they will die. Thus a fish AFP-mediated enhancement of ice nucleation as found by some researchers is unlikely to be adaptive. Our results show that enhancement of INP activity was not found at all INP and type III AFP concentrations in any case.

In contrast to the results with the fish AFP, when *Lp*AFP was added to INP preparations, the nucleation temperature was reliably decreased (Table 1; Fig. 2B). Remarkably, when *Lp*AFP (2 mg/ml) was added to INP (50 µg/ml), there was a mean freezing point depression of 1.9 ± 0.34 °C. This temperature is substantially greater than the recorded maximum TH activity of 0.45 °C for this protein [34] indicating that its "anti-nucleator" activity is greater than its ability to depress the freezing point. As well, the extent of the freezing point depression indicates that *Lp*AFP was not just adsorbing to embryonic ice crystals.

The observation that a consistent decrease in INA was achieved with an AFP found naturally in the same environment as the bacterial INP is noteworthy. Certain perennials have evolved several different cold-adaptive mechanisms, including cold-induced cell wall modification proteins, pathogenesis-related (PR) proteins to combat psychrophilic pathogens, and AFPs to inhibit IR [35]. Some of the PR proteins have themselves been shown to possess

antifreeze activity, thought to be due to cross-adaptation [4,35]. Reports of similar motifs between AFPs and PR proteins, however, do not extend to the ice-binding regions of *Lp*AFP [12]. Therefore, we suggest that *Lp*AFP not only functions to prevent IR once freezing takes place, but may also serve to lower the high freezing temperature and corresponding large ice crystal sizes dictated by the near ubiquitous INP-producing bacteria. These microbes are not only found on the surface of the leaves [11], but also in the apoplast, the site of *L. perenne*'s AFP [16].

More difficult to understand is how INPs and *Lp*AFPs would interact. It was proposed that type III AFP inhibited ice nucleation via a complex interaction with ice crystals and foreign particles, such as dust [36], but given that we saw no freezing point depression with type III AFPs, coupled with our observation that *Lp*AFP decreases the INP-*Lp*AFP solution freezing point more than its TH value, this model is not satisfactory. Rather, we speculate that some *Lp*AFP molecules can associate with the flat, repetitive ice nucleation sites on the INPs. These large proteins have been suggested to assemble in an overlapping, 'stair-like' fashion on the surface of bacterial cells since ice may preferentially form on steps [21]. If *Lp*AFP associated with even some of these flat surfaces, it could disrupt the aggregation of INP clusters at high subzero temperatures, thus preventing the formation of water clusters with a critical radius that would facilitate the further propagation of ice [37].

A physical interaction between *Lp*AFP and bacterial INPs has not previously been suggested, but the striking structural similarities including two comparable flat, relatively hydrophobic faces on opposite sides of both their respective ß-roll structures [38,39] could facilitate this. The parallel between the two distinct ice-associating proteins was convincingly demonstrated by the appearance of a ice crystal exhibiting a AFP-like inhibition morphology in experiments using a recombinant peptide fragment representing ⩽8% of the *P. syringae* INP [40]. Our observations (Fig. 3) showing GFP-labeled *Lp*AFP localized to the poles of *P. syringae* and *P. borealis* where INPs appear to cluster [22] helps support a physical interaction hypothesis. Interestingly, X-ray crystallographic analysis of purified *Lp*AFP show intermolecular interaction between *Lp*AFPs themselves along the β-roll surface [38] and thus it is possible that *Lp*AFP and INP could also interact. In contrast to the two-flat sided β-roll structure of *Lp*AFP, type III is globular [41]; presumably then, there would be no structural theoretical basis for a direct interaction of the non-plant AFP with INPs. Although the appearance of fluorescence on *Pseudomonas* was repeatable with the two species of ice nucleating bacteria, it was not always seen. These experiments were challenging not only because not all bacteria within a given strain produce INPs, but also likely because *Lp*AFP-GFP itself did not reduce INA activity as much as the wild-type protein (Table 1). We speculate that the presence of the GFP-tag reduced the affinity of the protein to the INP, as seen previously with another ice-like substrate [19].

Although the INP's INA was decreased in the presence of *Lp*AFP, there was no impact on any of the ice-binding activities of any of the tested AFPs. The absence of notable INP-mediated changes to IR inhibition, TH levels, or ice morphologies characteristic of each AFP indicates that INPs do not compete with AFPs for ice adsorption. Our hypothesis of a physical interaction between *Lp*AFP and INPs is consistent with these observations due to the large discrepancy in the size of the two proteins. INP monomers are more than 12-fold larger than *Lp*AFP. Perhaps only a single molecule of *Lp*AFP is enough to 'spoil' the ice nucleation surface of the INP. In turn, if these 'spoiled' INPs were then less likely to form ordered INP-INP aggregates, this could result in a significant depression of the INP-mediated freezing point as demonstrated in our experiments. Is it by chance or evolutionary design that only one of the two flat sides of *Lp*AFP binds ice [17], hypothetically allowing *Lp*AFP to 'spoil' INA without interfering with the active ice-binding site?

Regrettably, "a-side" (ice adsorption side) mutations could not be tested since they cannot be purified by ice-affinity. However, our results involving non-ice binding "b-side" *Lp*AFP mutants support our proposed model of INP inhibition. The freezing point depression clearly seen with wild-type *Lp*AFP was abolished when a single steric mutation (N72Y) was introduced on the b-side of *Lp*AFP's β-roll structure. A secondary steric mutation, T43Y, on the b-side modestly depressed the freezing point of *Lp*AFP, but this particular residue is located near the N-terminus at a bulge that interrupts the flat face, suggesting that the interaction between *Lp*AFP and INPs involves the flat surface [38]. Taken together, our results suggest that a freeze-tolerant perennial grass with *Lp*AFP should suffer less freeze damage, both by inhibition of IR as well as the lowering of the freezing point and initiating small ice crystal size, and together these activities will help ensure its winter survival.

Acknowledgments

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