Biochemical and Biophysical Research Communications 452 (2014) 636–641

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Perturbation of bacterial ice nucleation activity by a grass antifreeze protein

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ARTICLE INFO

Article history:
Received 19 August 2014
Available online 1 September 2014

Keywords:
Ice nucleation proteins
Antifreeze proteins
Ice binding proteins
Plant pathogen defence

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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http://dx.doi.org/10.1016/j.bbrc.2014.08.138
0006-291X/

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

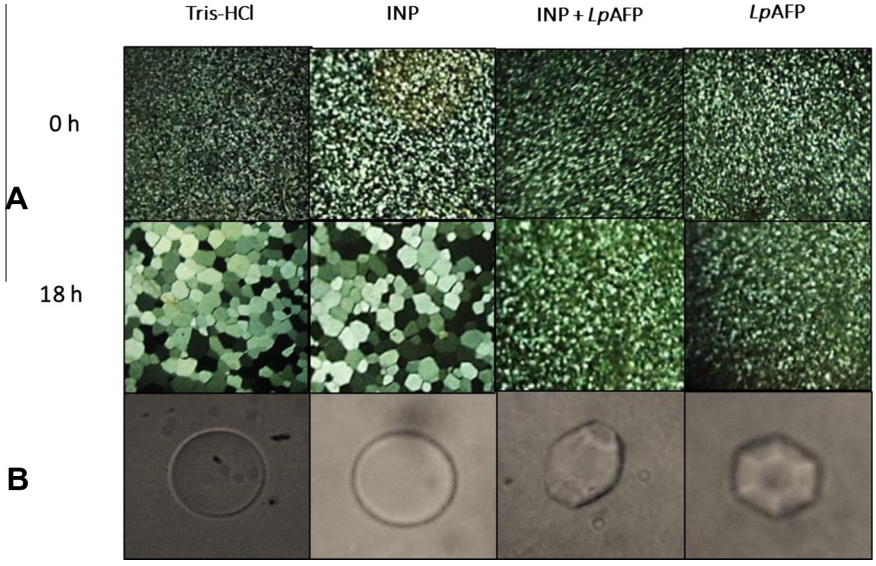

Fig. 1. Representative ice crystals from an IR inhibition splat assay (A) and typical ice crystal morphologies (B) in the presence of *P. syringae* ice nucleation protein (INP; 0.05 mg/ml) and *L. perenne* AFP (*Lp*AFP; 1 mg/ml). Both assays were performed in triplicate.