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Antifreeze Protein Produced Endogenously in Winter Rye Leaves[1]

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ABSTRACT

After cold acclimation, winter rye (*Secale cereale* L.) is able to withstand the formation of extracellular ice at freezing temperatures. We now show, for the first time, that cold-acclimated winter rye plants contain endogenously produced antifreeze protein. The protein was extracted from the apoplast of winter rye leaves, where ice forms during freezing. After partial purification, the protein was identified as antifreeze protein because it modified the normal growth pattern of ice crystals and depressed the freezing temperature of water noncolligatively.

Overwintering plants survive freezing temperatures by forming ice within their tissues. In these plants, it has generally been believed that ice formation occurs spontaneously in large vessels in plant tissues and then spreads throughout the plant at a rate determined by the environmental temperature (14). However, it has been clearly demonstrated in other frost-tolerant organisms such as insects, reptiles, and mollusks that the process of ice formation is controlled by specific proteins. These organisms all produce proteins that function as ice nucleators to initiate extracellular ice formation (6, 15, 19). Moreover, freezing-tolerant insects also produce antifreeze proteins that modify the ice crystals that form outside the cells (6).

We hypothesized that frost-tolerant plants may also produce proteins that control extracellular ice formation. We chose to conduct our experiments using winter rye (*Secale cereale* L.), an overwintering, herbaceous monocot that can survive temperatures below −20°C (11). It has been shown by scanning EM that cold-acclimated winter rye leaves survive freezing temperatures by forming ice only in intercellular spaces (17). In the experiments reported here, we examined cold-acclimated rye leaves for the capacity to modify ice in a manner similar to that observed for antifreeze proteins purified from arctic and antarctic fish (5) and frost-tolerant insects (6).

To prove that antifreeze proteins exist in plants, we must demonstrate that plant extracts possess two characteristics shown to be unique to previously purified antifreeze proteins. The first characteristic of antifreeze proteins is a distinctive, concentration-dependent effect on ice crystal morphology (5). In pure water, ice normally grows parallel to the basal plane (*a* axes) of the crystal lattice, with little growth perpendicular to the basal plane (the *c* axis), so that the ice crystals appear flat and round. Low concentrations (nM) of antifreeze proteins preferentially inhibit the growth of ice along the *a* axes so that the hexagonal prism faces of the crystal become evident. At higher concentrations (μM) of antifreeze protein, the crystals grow predominantly along the *c* axis to form hexagonal bipyramids and needle-like crystals (5). The second unique characteristic of antifreeze proteins is a measurable degree of thermal hysteresis. Thermal hysteresis, the difference between freezing and melting temperatures, is determined by observing the effect of temperature on the growth of a single ice crystal. On the one hand, antifreeze proteins lower the Tm of the solution by colligative effects. On the other hand, antifreeze proteins lower the freezing temperature of a solution more than its melting temperature by noncolligative effects, i.e. by binding to prism faces and inhibiting ice crystal growth (5). We now show that ice crystals formed in apoplastic extracts obtained from frost-tolerant winter rye leaves exhibit changes in morphology and thermal hysteresis that are consistent with the defined activity of antifreeze proteins.

MATERIALS AND METHODS

Plant Material

Winter rye (*Secale cereale* L. cv Musketeer) seeds were planted in coarse vermiculite in 15-cm pots, which were maintained in growth chambers with a day/night temperature regimen of 20/16°C, a daylength of 16 h, and an average PPFD of 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$. After 7 d, the plants were either maintained at 20/16°C for another 21 d (nonacclimated) or transferred to 5/2°C for 49 d to induce cold acclimation. Plants were watered as required with modified Hoagland solution (10). Under these growth conditions, leaves from nonacclimated plants withstand freezing to −12°C, whereas cold-acclimated leaves tolerate −22°C (11).

Apoplastic Protein Extraction

Proteins were extracted from the leaf apoplast as described by Mauch and Staehelin (16). Leaves (7 g fresh weight) were cut into 2-cm lengths, rinsed in three changes of distilled water to remove cell contents from cut surfaces, and vacuum-

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infiltrated for 20 min with 5 mM EDTA, 10 mM ascorbic acid, 10 mM mercaptoethanol, 1 mM PMSF, 2 mM caproic acid, and 2 mM benzamidine. The leaves were patted dry and inserted into a 20-mL syringe, which was placed, in turn, in a 50-mL centrifuge tube. After centrifugation for 20 min at 830*g*, the apoplastic extract was recovered from the bottom of the tube. Extracts containing visible levels of Chl were discarded at this step. Proteins were assayed using the Bradford assay with BSA as the standard (2).

Column Chromatography

Apoplastic extracts were concentrated 5-fold and exchanged into 50 mM NH_4HCO_3 by ultrafiltration (Centriprep-10; Amicon Canada Ltd., Oakville, Ontario, Canada). An aliquot containing 250 μg of protein was applied to a Sephacryl 200 (Pharmacia LKB Biotechnology, Uppsala, Sweden) column (0.5 × 32 cm) in 50 mM NH_4HCO_3. The eluate was monitored initially for UV absorbance at 280 nm, although absorbance at 230 nm was measured later using a UV-visible spectrophotometer. Fractions exhibiting absorbance at 280 nm were assayed for antifreeze activity. Fractions containing antifreeze activity were pooled, freeze-dried, and resuspended in a minimal volume of distilled water.

Antifreeze Activity

Antifreeze activity was assayed using a nanoliter osmometer (Clifton Technical Physics, Hartford, NY) in which the freezing stage was mounted on the stage of a phase contrast light photomicroscope (Olympus BHT; Carsen Medical and Scientific Co., Markham, Ontario, Canada) so that the morphology of individual ice crystals formed in solution could be observed (3). Freezing temperature and Tm were calculated using the osmometer (°C = [milliosmol × 1.86°C]/1000). The Tm was taken when the faces of an ice crystal became round; the freezing temperature was taken when the ice crystal elongated along its *c* axis in the form of needles (see Fig. 3D).

Protease Treatment

Apoplastic extracts from cold-acclimated rye leaves were incubated with 5% (w/v) *Streptomyces griseus* protease (Sigma) at 22°C.

Protein Electrophoresis

Proteins were solubilized, separated by SDS-PAGE (13) on 13.5% polyacrylamide gels (7 × 7 × 0.15 cm) and visualized using silver stain (21).

RESULTS

Antifreeze Activity in Apoplastic Extracts

Ice crystals formed in apoplastic extracts of nonacclimated rye leaves were slightly hexagonal (Fig. 1B) with no discernible *c* axis growth or measurable thermal hysteresis. The fact that the crystals were slightly hexagonal indicates that the apoplastic extracts of nonacclimated rye leaves contain the minimal amount of antifreeze activity that can be detected by this technique.

In contrast, all apoplastic extracts of cold-acclimated winter rye leaves formed hexagonal ice crystals upon freezing (Fig. 1, C-G). As the temperature was lowered, these crystals expanded first along the *c* axis to form incomplete hexagonal bipyramids (Fig. 1C) and then along the *a* axes to form both hexagonal columns (Fig. 1D) and larger hexagonal plates of ice (Fig. 1, E-G). The formation of hexagonal ice and the growth of ice crystals along the *c* axis are both indicative of antifreeze activity in these crude, apoplastic extracts of cold-acclimated winter rye leaves.

When apoplastic extracts from cold-acclimated rye leaves were incubated with protease for 20 min, the antifreeze

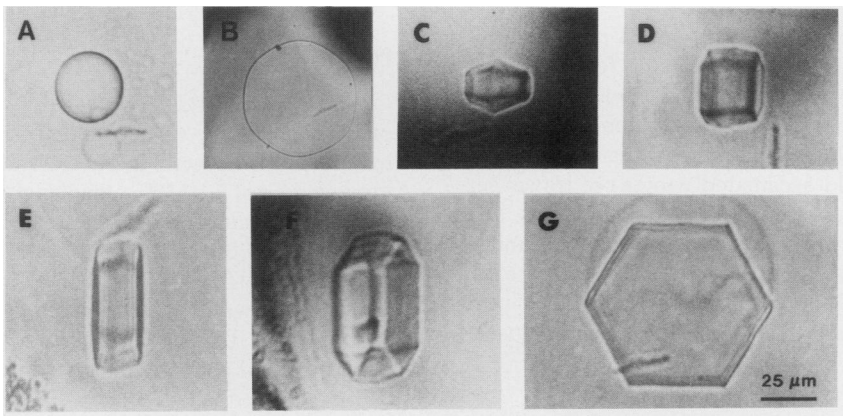

Figure 1. Antifreeze activity in apoplastic extracts of winter rye leaves. Antifreeze activity was determined by observing ice crystal morphology using a nanoliter osmometer. A, Ice crystal formed in distilled water, oriented so that the basal plane is parallel to the plane of the page. All prism faces of the crystal are round. B, Ice crystal formed in an apoplastic extract from nonacclimated winter rye leaves. Although the prism faces are slightly expressed, there is no visible growth of the crystal along the c axis (normal to the plane of the page). C–F, Growth sequence of an ice crystal as the temperature was lowered in an apoplastic extract from cold-acclimated winter rye leaves. Hexagonal prism faces and growth of the crystal along the c axis (oriented parallel to the plane of the page) are clearly visible. G, Hexagonal column of ice as shown in F, oriented so that the c axis is normal to the plane of the page.

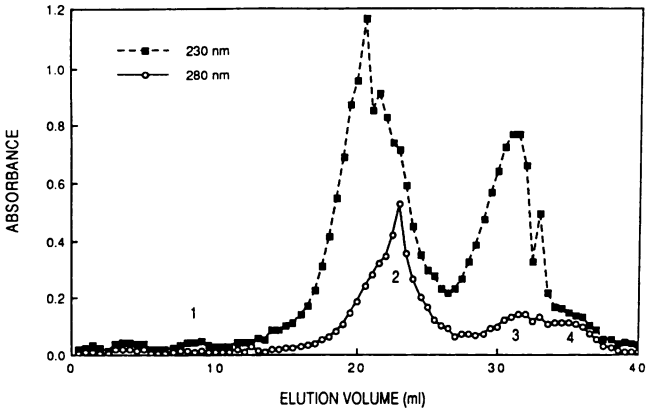

Figure 2. Fractionation of apoplastic extracts from cold-acclimated leaves by column chromatography. Although there were four peaks of absorbance at 280 nm, antifreeze activity was found only in fractions associated with peak 2. Fractions eluting at 17.5 to 21.0 mL formed bipyramidal crystals upon freezing and also exhibited very low absorbance at 280 nm when compared with absorbance at 230 nm.

activity was reduced. Instead of forming partially bipyramidal crystals, the extracts formed hexagonal plates. After a 1-h incubation with protease, all antifreeze activity disappeared and only thin, round crystals, characteristic of crystals formed in distilled water (Fig. 1A), were observed. These results support the conclusion that antifreeze activity in winter rye is derived from protein.

Partial Purification of Antifreeze Protein

Apoplastic extracts of cold-hardened rye leaves were concentrated to 1.6 mg protein·mL^{-1} by ultrafiltration and then fractionated on a Sephacryl 200 column. The elution profile revealed four peaks of absorbance at 280 nm (Fig. 2). Fractions from peaks 1, 3, and 4 exhibited no observable effects on ice crystal morphology, because only round crystals were observed. In contrast, fractions from the second peak significantly altered ice crystal morphology. Fractions eluting at 17.5 to 21.0 mL formed bipyramidal ice crystals in the antifreeze assay, whereas fractions eluting at 21.1 to 26.5 mL formed only hexagonal ice crystals. The protein present in peak 2 exhibited low absorbance at 280 nm, when compared with absorbance at 230 nm (Fig. 2), as has been observed for fish antifreeze proteins because they contain few aromatic amino acids (5).

Thermal Hysteresis

Column fractions exhibiting the capacity to form bipyramidal ice crystals (fractions eluting at 20.0–21.0 mL in peak 2) were pooled, lyophilized, and resolubilized in a minimal volume of distilled water for the determination of thermal hysteresis. At this higher protein concentration (60 mg·mL^{-1}), ice crystal growth was inhibited along the *a* axes (Fig. 3, B-D). Furthermore, the ice crystals spiked along the *c* axis (Fig. 3D) at an average freezing temperature of −1.10°C for five ice crystals. The average Tm was −0.78°C, so the thermal hysteresis was calculated to be 0.33 ± 0.06°C (mean ± SD, n = 5). The thermal hysteresis exhibited by the winter rye antifreeze protein is smaller than that observed for antifreeze proteins found in polar fish (approximately 0.6°C [7]) or in insects (5°C [6, 7]).

Polypeptide Composition of Column Fractions

As shown by SDS-PAGE, fractions from peak 2 that exhibit the capacity to form bipyramidal crystals contain six major polypeptides ranging in size from 9 to 36 kD (Fig. 4, lane 4). We have not yet isolated these polypeptides, nor have we determined which of these polypeptides exhibits antifreeze activity. It should be noted, however, that fish from polar regions synthesize as many as eight polypeptides or glycopeptides that exhibit antifreeze activity (4, 7), so it is possible that more than one of these polypeptides may have antifreeze activity.

DISCUSSION

The presence of an endogenous antifreeze protein in plants was proposed by Kurkela and Franck (12) after they cloned a cold-inducible gene from *Arabidopsis* that exhibited some

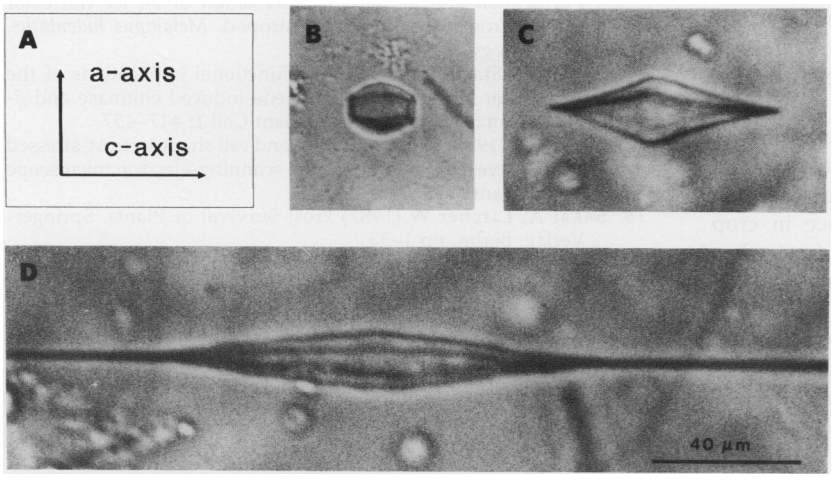

Figure 3. Ice crystal morphology of partially purified and concentrated antifreeze protein from cold-acclimated winter rye leaves. A, Orientation of the ice crystals. The *a* axis represents growth in the basal plane and the c axis represents growth normal to the basal plane. B–D, Growth sequence of an ice crystal as the temperature was lowered. B, Incomplete bipyramid; C, bipyramid; D, needle-like.

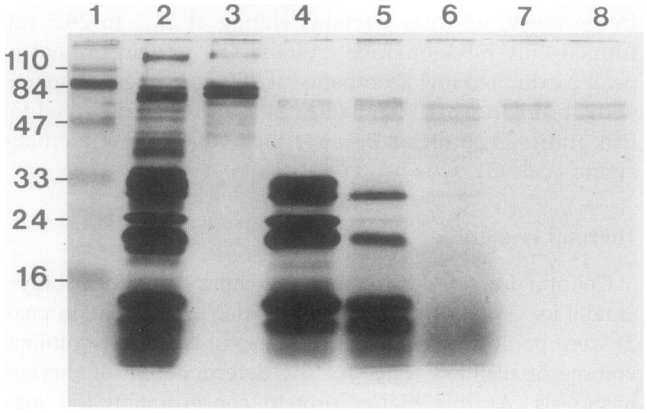

Figure 4. SDS-PAGE of polypeptides associated with column fractions of each 280-nm peak shown in Figure 2. The 13.5% acrylamide gel was silver stained. Lane 1, Prestained molecular mass standards (kD); lane 2, crude intercellular extract; lane 3, polypeptides eluted at 8 mL (peak 1); lane 4, polypeptides eluted at 18 mL (shoulder of peak 2); lane 5, polypeptides eluted at 22 mL (peak 2); lane 6, polypeptides eluted at 26 mL (shoulder of peak 2); lane 7, polypeptides eluted at 31 mL (peak 3); lane 8, polypeptides eluted at 35 mL (peak 4).

homology to the gene encoding the winter flounder antifreeze protein. We have now demonstrated that apoplastic extracts of winter rye leaves exhibit both a concentration-dependent ability to modify the normal growth pattern of ice (Figs. 1 and 3) and the capacity to depress the freezing temperature of a solution noncolligatively. Because these characteristics meet the definition of the activity of an antifreeze protein (5), we conclude that winter rye leaves produce antifreeze protein that is secreted into the apoplast during cold acclimation. Thus, there are biological factors, as well as environmental factors, that influence ice formation in plants.

Ice formation is an event that takes place at relatively warm subzero temperatures in plants. Although the presence of antifreeze protein may distinguish a frost-sensitive plant from a frost-tolerant plant, antifreeze protein may not be the factor that determines the lower limit of cell survival in cold-acclimated tissues subjected to freezing temperatures. As temperatures decrease, intracellular water is lost to the growing extracellular ice masses and the cells themselves become dehydrated (17, 18). As a result, the lowest temperature at which frost-tolerant plants survive is likely to be correlated with the desiccation tolerance of the cells (20).

In the future, the discovery of antifreeze protein intrinsically produced by a frost-tolerant plant may prove important to agriculture for two reasons. First, conventional breeding programs have failed to improve frost resistance in crop plants because physiological markers specific for frost tolerance are not yet available (1). The antifreeze protein reported here is the first protein demonstrated to be directly involved in the process of ice formation in plants and may prove useful as a selection marker for crop improvement programs. Second, isolation and characterization of gene(s) for antifreeze protein may provide the targeting information essential for the successful transformation of freezing-sensitive crop plants with genes encoding antifreeze proteins (8, 9).

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