Plant Physiol. (1996) 110: 845–857

Immunolocalization of Antifreeze Proteins in Winter Rye Leaves, Crowns, and Roots by Tissue Printing[1]

Mervi Antikainen*, Marilyn Griffith, Jing Zhang, Wai-Ching Hon, Daniel S. C. Yang, and Kaarina Pihakaski-Maunsbach

Laboratory of Plant Physiology and Molecular Biology, Department of Biology, FIN-20014 University of Turku, Finland (M.A., K.P.-M.); Department of Biology, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1 (M.G., J.Z.); and Department of Biochemistry, McMaster University, Hamilton, Ontario, Canada L8N 3Z5 (W.-C.H., D.S.C.Y.)

During cold acclimation, antifreeze proteins (AFPs) that are similar to pathogenesis-related proteins accumulate in the apoplast of winter rye (*Secale cereale* L. cv Musketeer) leaves. AFPs have the ability to modify the growth of ice. To elucidate the role of AFPs in the freezing process, they were assayed and immunolocalized in winter rye leaves, crowns, and roots. Each of the total soluble protein extracts from cold-acclimated rye leaves, crowns, and roots exhibited antifreeze activity, whereas no antifreeze activity was observed in extracts from nonacclimated rye plants. Antibodies raised against three apoplastic rye AFPs, corresponding to a glucanase-like protein (GLP, 32 kD), a chitinase-like protein (CLP, 35 kD), and a thaumatin-like protein (TLP, 25 kD), were used in tissue printing to show that the AFPs are localized in the epidermis and in cells surrounding intercellular spaces in cold-acclimated plants. Although GLPs, CLPs, and TLPs were present in nonacclimated plants, they were found in different locations and did not exhibit antifreeze activity, which suggests that different isoforms of pathogenesis-related proteins are produced at low temperature. The location of rye AFPs may prevent secondary nucleation of cells by epiphytic ice or by ice propagating through the xylem. The distributions of pathogenesis-induced and cold-accumulated GLPs, CLPs, and TLPs are similar and may reflect the common pathways by which both pathogens and ice enter and propagate through plant tissues.

Overwintering plants such as winter rye (*Secale cereale* L.) survive freezing temperatures by forming ice in intercellular spaces and in xylem tracheids and vessels within their tissues (Pearce, 1988; Guy, 1990; Griffith and Antikainen, 1996). During cold acclimation, winter rye leaves produce intrinsic ice nucleators that have been shown to initiate the formation of extracellular ice during freezing under controlled conditions (Brush et al., 1994). Moreover, winter rye has been shown to accumulate AFPs, which have the ability to modify the growth of ice (Griffith et al., 1992; Marentes et al., 1993). AFP secretion in winter rye is associated with increasing freezing tolerance, which reaches −23°C after 7 weeks of cold acclimation at 5/2°C (day/night) with an 8-h day (Brush et al., 1994). AFPs also accumulate in other freezing-tolerant organisms, including insects and other terrestrial arthropods as well as both nonvascular and vascular plants (Duman et al., 1993). The function of AFPs in freezing-tolerant organisms may be to inhibit ice recrystallization during freezing and thawing cycles (Knight and Duman, 1986).

Six AFPs have been isolated and characterized from the apoplast of winter rye leaves (Hon et al., 1994). These proteins were identified as GLPs, CLPs, and TLPs (Hon et al., 1995). Similar proteins, endo-β-1,3-glucanases, endochitinases, and TLPs, are known to be related to the mechanism for plant disease resistance (Carr and Klessig, 1989; Bol et al., 1990; Collinge et al., 1993; Stintzi et al., 1993) and are therefore classified as PR proteins. Proteins similar to PR proteins are also induced by other conditions unrelated to pathogen induction, such as ethylene (Boller et al., 1983), UV light (Yalpani et al., 1994), ozone (Ernst et al., 1992; Kärenlampi et al., 1994; Yalpani et al., 1994), high salt (Esaka et al., 1994), heat (Margis-Pinheiro et al., 1994), phytotoxic metals (Jacobsen et al., 1992), and wounding (Zhang and Punja, 1994). Cold-induced accumulation of chitinase has been observed in barley (Tronsmo et al., 1993) and bermudagrass (Gatschet et al., 1996). Moreover, low temperature has been shown to increase accumulation of three mRNAs in potato that correspond to transcripts of cDNAs encoding osmotin-like proteins (Zhu et al., 1995). These proteins are also known as PR-5 proteins and are similar to TLPs (Zhu et al., 1995). Although a 67-kD AFP was purified recently from the bittersweet nightshade *Solanum dulcamara*, this protein showed no similarity to other proteins (Duman, 1994).

Little information exists concerning the location and function of proteins preferentially synthesized at low temperature (Guy, 1990; Palva, 1994). To learn more about the ice-binding function of winter rye AFPs, it is important to

[1] This work was supported by grants from the Natural Science and Engineering Research Council of Canada to M.G., the Canadian Space Agency to D.S.C.Y., the Academy of Finland to K.P.-M., and the fellowships from the Jenny and Antti Wihuri Foundation and the Turku University Foundation to M.A.

* Corresponding author; e-mail mervi.antikainen@utu.fi; fax 358–21–3335549.

Abbreviations: AFP, antifreeze protein; BCIP, 5-bromo-4-chloro-3-indolylphosphate-toluidine salt; CA, cold-acclimated; CLP, chitinase-like protein; GLP, β-1,3-glucanase-like protein; NA, nonacclimated; NBT, nitroblue tetrazolium; PR protein, pathogenesis-related protein; TLP, thaumatin-like protein.

know where these proteins accumulate in CA tissue. Although winter rye AFPs have been extracted from the leaf apoplast (Griffith et al., 1992), their presence in other cellular compartments or their tissue specificity has not been determined. In this paper, we report, to our knowledge for the first time, the localization of AFPs in plant tissues. First, we extracted total soluble proteins from CA rye leaves, crowns, and roots and demonstrated that each of these extracts exhibits antifreeze activity. Second, we purified three apoplastic AFPs corresponding to a GLP, a CLP, and a TLP, raised antibodies against them, and showed by tissue printing that GLPs, CLPs, and TLPs are differentially localized in NA and CA winter rye plants.

MATERIALS AND METHODS

Plant Material

Winter rye (*Secale cereale* L. cv Musketeer) seedlings were planted in coarse vermiculite and grown in a controlled environment growth chamber at 20/16°C (day/night), with a 16-h photoperiod and a PPFD of 300 μmol photons m^{-2} s^{-1}. After 7 d, some of the plants were acclimated to cold by transferring them to low temperature (5/2°C; day/night) with a short daylength (8 h) for at least 7 weeks. NA plants were maintained at 20/16°C with a 16-h photoperiod for 3 weeks. Plants were watered as required with modified Hoagland solution (Huner and Macdowall, 1976).

Extraction of Apoplastic Proteins and Total Soluble Proteins

Apoplastic proteins were extracted from NA and CA winter rye leaves by vacuum infiltration with 20 mM ascorbate and 20 mM $CaCl_2$ (pH 3), followed by centrifugation to recover the infiltrate, as described by Hon et al. (1994). Total soluble proteins were obtained from leaves, crowns, and roots and from leaves where the apoplastic proteins had been extracted, according to the method of Kurkela et al. (1988). Rye tissues (1.0 g) were frozen in liquid nitrogen and ground into a powder, which was homogenized in an extraction buffer (50 mM Tris-HCl, pH 6.8, 1% β-mercaptoethanol, 50 mg L^{-1} PMSF). Soluble protein-containing supernatants were collected after centrifugation at 15,800*g* and 4°C for 5 min. Protein concentrations were measured using a modified Bradford (1976) procedure (Bio-Rad Laboratories Ltd.) with BSA as the standard protein. Apoplastic extract from NA winter rye leaves was concentrated by ultrafiltration (Ultrafree-MC 10,000 NMWL filters; Millipore).

Assay of Antifreeze Activity

Antifreeze activity in leaf apoplastic extracts and in total soluble protein extracts from leaves, crowns, and roots was assayed using a Clifton nanoliter osmometer (Clifton Technical Physics, Hartford, NY) and a phase-contrast photomicroscope (Olympus BHT) as described in detail by Hon et al. (1994). Antifreeze activity was determined qualitatively by observing the morphology of ice crystals growing in solution. In this system, ice crystals grown in water are disc-shaped. Assays of fish AFPs have shown that ice crystals grown in a dilute solution (nanomolar) of AFP form hexagonal discs, crystals grown in moderate concentrations (micromolar) of AFPs form hexagonal columns, and crystals grown in high concentrations (micromolar to millimolar) of AFPs form hexagonal bipyramids (DeVries, 1986).

Purification of GLPs, TLPs, and CLPs

GLP and TLP were purified as denatured polypeptides by preparative electrophoresis. Apoplastic proteins were extracted from the leaves of winter rye plants that had been cold-acclimated for 7 weeks with a 16-h daylength. Proteins were precipitated from an apoplastic extract containing 10 mg of proteins using 40%, followed by 70%, saturated $(NH_4)_2SO_4$ at 0°C. The precipitate was dissolved in 2 mL of distilled H_2O and dialyzed overnight against several changes of H_2O at 4°C. After dialysis, an equal volume of sample buffer was added, and the sample was heated at 95°C for 5 min (Laemmli, 1970). For the isolation of GLP, apoplastic polypeptides were separated using the Prep Cell (model 491, Bio-Rad) with a 13% polyacrylamide separating gel. Fractions (2.5 mL) were collected and examined by SDS-PAGE using 12% polyacrylamide gels and silver stain. Fractions containing only the 32-kD GLP were pooled. For the isolation of TLP, preparative electrophoresis was conducted using a 15% polyacrylamide separating gel. Fractions containing the 25-kD TLP were pooled. Protein concentrations were assayed using the Lowry assay (Lowry et al., 1951), as modified by Bio-Rad (detergent-compatible protein assay), with BSA as a standard. CLP was purified in its native state by affinity chromatography using colloidal chitin (Huynh et al., 1992). Briefly, apoplastic extracts from CA rye leaves were dialyzed against 20 mM NH_4HCO_3, pH 8.0, and stirred with colloidal chitin. The colloidal chitin was washed with 20 mM NH_4HCO_3, pH 5.2, to remove nonspecifically bound proteins before CLP (35 kD) was eluted with 20 mM acetic acid, pH 3.0 (Hon et al., 1995).

The identities of the purified GLP, CLP, and TLP were confirmed by immunoblotting as described by Hon et al. (1994). Rabbit anti-basic β-1,3-glucanase IgG (Keefe et al., 1990), anti-endochitinase antiserum (Legrand et al., 1987), and anti-TLP antiserum (Pierpoint et al., 1992), all of which were produced against proteins induced by pathogens in tobacco, were used as the primary antibodies at dilutions of 0.4 mg%, 1:5000, and 1:1000, respectively. The immunoreactions were detected by alkaline phosphatase conjugated to goat anti-rabbit IgG (Sigma) with BCIP (Sigma) and tetrazolium blue chloride (Sigma) used as substrates.

Production of Antisera

Antisera were produced against all three classes of the purified AFP. For the 32-kD GLP that accumulates at low temperature, the rabbits were immunized with 250 μg of the denatured polypeptide emulsified with Freund's complete adjuvant (Sigma). The first booster injection was given after 6 weeks using 100 μg of the denatured polypep-

tide emulsified with Freund's incomplete adjuvant (Sigma). The rabbits were bled 3 weeks later and then at 4-week intervals. The antiserum titer was 1:62,500.

Antiserum was produced against the winter rye 25-kD TLP that accumulates at low temperature by immunization of rabbits with 100 μg of the denatured polypeptide emulsified in Freund's complete adjuvant, followed in 6 weeks by the subcutaneous injection of 50 μg of the denatured polypeptide emulsified in Freund's incomplete adjuvant. The rabbits were bled 6 weeks later, and the antiserum titer was 1:12,500.

For preparation of antiserum against the native 35-kD CLP, 0.48 mg of purified protein was emulsified with 0.5 mL of Freund's complete adjuvant and injected into a rabbit. The first and second booster injections were given on d 26 and 32, using 0.48 and 1.9 mg of CLP, respectively. A blood sample was withdrawn on d 67. The antiserum titer was 1:5000.

The specificities of the three antisera were tested using leaf apoplastic extract. The proteins were solubilized, separated by SDS-PAGE (15% polyacrylamide), and transferred onto nitrocellulose as described below. Antisera against winter rye GLP and TLP were used in dilutions of 1:10,000, and a 1:1,000 dilution was used in the case of anti-CLP antiserum.

SDS-PAGE and Immunoblotting

Equal amounts of apoplastic and total soluble polypeptides were separated on a 15% SDS-polyacrylamide gel and stained with Coomassie brilliant blue (Laemmli, 1970). Electroblotting and immunodetection were performed according to the method of Ghosh et al. (1989) with modifications. Separated polypeptides and prestained low-molecular-mass markers (Bio-Rad) were electroblotted onto a nitrocellulose membrane (Bio-Rad) using the Mini Trans-Blot (Bio-Rad) as recommended by the supplier. Blots were blocked overnight in a buffer containing 5% skim milk powder and 0.01% Tween 20, followed by 2 h of incubation with either the anti-GLP antiserum (dilution 1:500) or the anti-TLP antiserum (dilution 1:500), or overnight with the anti-CLP antiserum (dilution 1:500). The color reaction was detected after a 2-h incubation with alkaline phosphatase conjugated to goat anti-rabbit secondary antibody using BCIP and NBT (Sigma) as substrates.

Light Microscopy

The anatomy of winter rye leaves, crowns, and roots was examined by light microscopy. Freehand cross-sections were obtained using a double-edged razor blade and stained with phloroglucinol hydrochloride, which stains lignin. Root sections were obtained 1 to 1.5 cm from the root tips of both NA and CA plants. The crown was sectioned near the root-shoot junction. Fully expanded leaves were sectioned from mid-length regions. These sections were vacuum infiltrated in 50% ethanol for 15 to 20 min prior to staining to avoid trapping air bubbles in the mesophyll. Sections were examined with a Zeiss Axiophot Photomicroscope (Carl Zeiss Canada) and Leitz Orthoplan microscope (Leitz, Wetzlar, Germany).

Tissue Printing

Tissue-specific localization of GLP, CLP, and TLP was determined by blotting proteins onto nitrocellulose membranes followed by immunological detection with specific antibodies. Winter rye tissues for tissue printing were sectioned freehand with double-edged razor blades. Leaf tissues were placed in a slit in a Styrofoam block to support leaf tissues during sectioning. Roots, crowns, and leaves were prepared as for light microscopy. Tissue printing was performed according to the method of Ye and Varner (1991) with slight modifications. Freshly cut surfaces were pressed to the nitrocellulose membrane (Bio-Rad; 0.45 μm pore size) for 15 to 30 s. Air-dried tissue prints were either stained with amido black (Sigma) to visualize total proteins or incubated with rabbit polyclonal antibodies against winter rye GLP (dilution 1:500), CLP (1:500), and TLP (1:500) to localize AFPs. Alkaline phosphatase-conjugated goat anti-rabbit IgG was used as a secondary antibody with a dilution of 1:3000, and BCIP and NBT were used for color development. Controls for secondary antibody were performed by replacing the primary antibody solutions with 5% skim milk powder. Tissue prints were photographed using a Zeiss Stemi SV II stereomicroscope (Carl Zeiss Canada) or a Wild Makroskop M 420 stereomicroscope (Wild, Heerbrugg, Switzerland).

RESULTS

Location of Antifreeze Activity in Winter Rye Plants

To determine the general distribution of AFPs in NA and CA winter rye plants, antifreeze activity was assayed in extracts of total soluble proteins from leaves, crowns, and roots and of apoplastic proteins from leaves. In addition, leaves were ground for total soluble protein extraction after removal of apoplastic proteins to determine whether some AFPs were localized intracellularly.

In NA plants, antifreeze activity was absent in extracts of leaves, crowns, and roots because only disc-shaped ice crystals formed in solution (Fig. 1, A, C, G, and I). Moreover, there was no antifreeze activity present in the leaf apoplast (Fig. 1A) or in leaves from which apoplastic proteins were extracted (Fig. 1E). In NA winter rye leaves, the protein concentration of the apoplastic extract was approximately 10 times more dilute than in CA leaves. However, we did not detect any antifreeze activity even after concentration of the NA apoplastic extract (Fig. 1A).

Total protein extracts obtained from leaves, crowns, and roots of CA plants formed complex ice crystals shaped like hexagonal columns (Fig. 1, D, F, H, and J). The formation of a hexagonal bipyramid was observed in the apoplastic protein extract obtained from CA leaves (Fig. 1B). However, antifreeze activity was also detected after extraction of the apoplastic proteins from CA leaves, indicating that either the extraction process was incomplete and/or some of the antifreeze activity was intracellular (Fig. 1F).

Purification of AFPs and Specificity of the Antisera

The six apoplastic AFPs that accumulated at low temperature were identified as two GLPs, two CLPs, and two

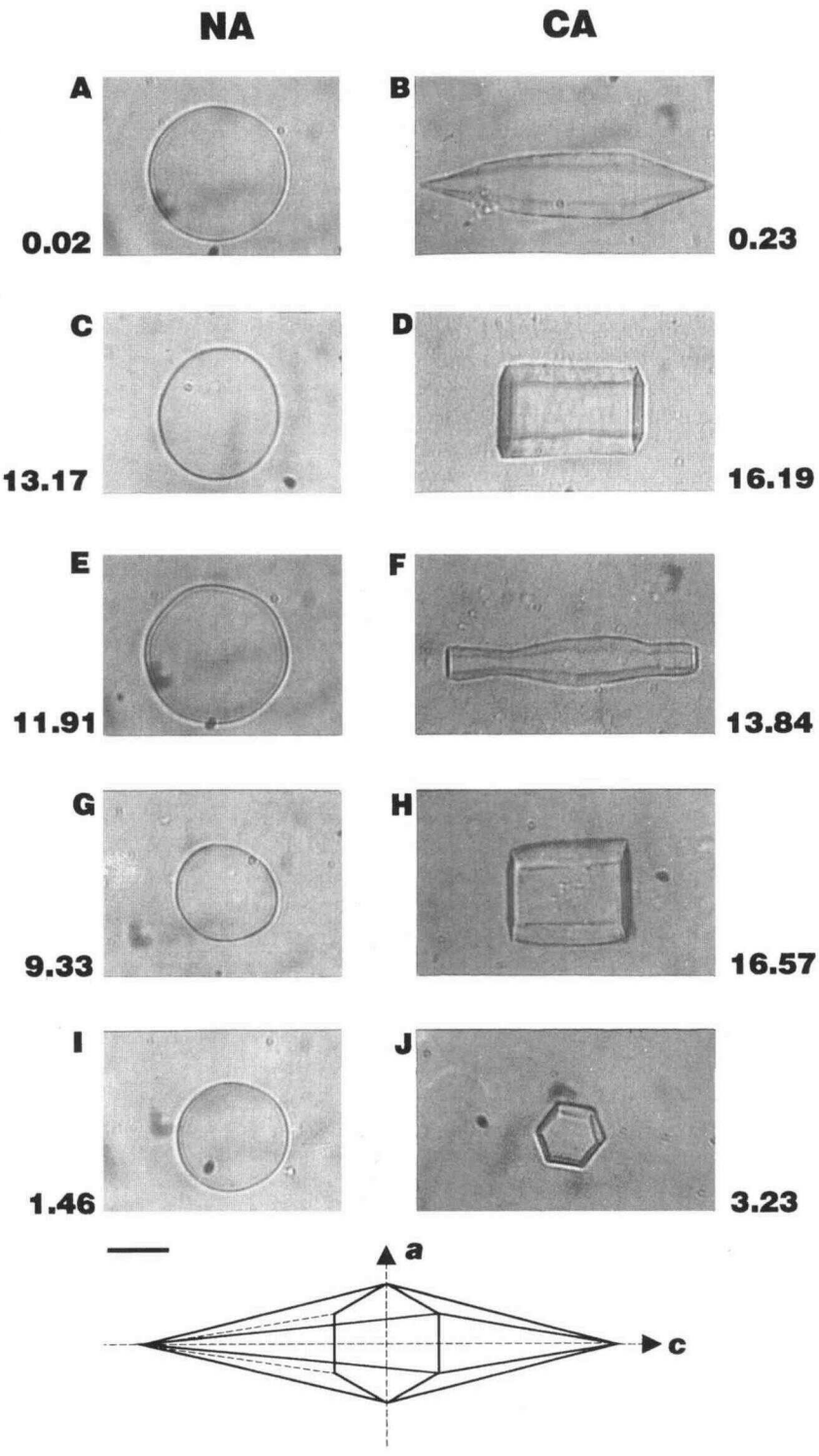

Figure 1. Antifreeze activity present in winter rye tissues as shown by representative ice crystals grown in extracts of NA (A, C, E, G, and I) and CA (B, D, F, H, and J) tissues. Soluble proteins were extracted from the leaf apoplast (A and B), intact leaves (C and D), leaves from which apoplastic proteins had been extracted (E and F), crowns (G and H), and roots (I and J). Numbers beside the figures represent the protein concentration of the original sample in mg g^{-1} fresh weight. In A, 0.02 mg g^{-1} fresh weight refers to the original concentration of NA leaf apoplastic protein extract even though the ice crystal shown was actually grown in a more concentrated solution (0.219 mg protein mL^{-1}) to ensure that the lack of antifreeze activity was not due to the low protein concentration. The diagram at the bottom of the figure shows the orientation of the *a* and *c* axes in an ice crystal growing along the *c* axis to form a hexagonal bipyramid. Ice crystal morphologies observed in protein solutions indicate differences in antifreeze activity: circular discs (A, C, E, G, and I) indicate no antifreeze activity, whereas hexagonal discs (J), columns (D, F, and H), and bipyramids (B) represent low, moderate, and high antifreeze activity, respectively. In ice crystals A, C, E, G, I and J, the *c* axis is perpendicular to the plane of the photograph. In ice crystals B, D, F, and H, the *c* axis is parallel to the plane of the photograph. Magnification bar = 17 μm.

TLPs (Hon et al., 1995). Antisera were raised against one of each type of AFP, namely the 32-kD GLP, 35-kD CLP, and 25-kD TLP (Fig. 2A). The specificities of the antisera used for the immunolocalization studies are shown in Figure 2, B, C, and D. Each of the polyclonal antibodies recognized only the corresponding type of proteins in the apoplastic extract from CA leaves. Anti-GLP antiserum raised against the 32-kD polypeptide, used in a dilution of 1:10,000, recognized a polypeptide with a molecular mass of 32 kD (Fig. 2B), which was identified as a GLP by Hon et al. (1995). With a dilution of 1:500, the anti-GLP antiserum also recognized a 35-kD polypeptide (Fig. 3D, lane 4) also known to be a GLP (Hon et al., 1995). Anti-CLP antiserum recognized only one 35-kD polypeptide at a dilution of 1:1,000

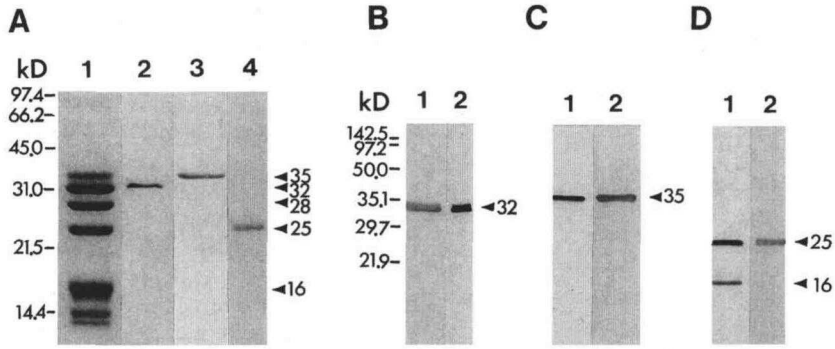

Figure 2. Specificity of antisera produced against purified AFPs from CA winter rye leaves. A, SDS-PAGE of purified AFPs from CA winter rye leaves. The gel (15% polyacrylamide) was stained with Coomassie brilliant blue. Lane 1, Apoplastic extract (20 μg of protein) from CA winter rye leaves containing all three classes (GLPs, CLPs, and TLPs) of AFPs; lane 2, 1 μg of purified GLP; lane 3, 1 μg of purified CLP; and lane 4, 1 μg of purified TLP. Bio-Rad low-molecular-mass markers are indicated on the left. Numbers on the right refer to the molecular masses of polypeptides present in apoplastic extract. B, Immunoblot probed with anti-GLP antiserum (dilution 1:10,000). Lane 1, Apoplastic extract (1 μg of protein) from CA winter rye leaves; lane 2, 1 μg of purified GLP. Bio-Rad prestained molecular mass markers are indicated on the left. C, Immunoblot probed with anti-CLP antiserum (dilution 1:1,000). Lane 1, Apoplastic extract (1 μg of protein) from CA winter rye leaves; lane 2, 1 μg of purified CLP. D, Immunoblot probed with anti-TLP antiserum (dilution 1:10,000). Lane 1, Apoplastic extract (1 μg of protein) from CA winter rye leaves; lane 2, 1 μg of purified TLP. Numbers on the right refer to the molecular masses of the purified and denatured AFPs.

(Fig. 2C) and recognized two polypeptides of 35 and 28 kD at a dilution of 1:500 (Fig. 3F, lane 4). The 35- and 28-kD polypeptides were both identified as CLPs by Hon et al. (1995). Antiserum raised against the 25-kD TLP detected two polypeptides with molecular masses of 25 and 16 kD (Fig. 2D), which were both identified as TLPs by Hon et al. (1995). In summary, each polyclonal antiserum was specific to one type of AFP and not to other apoplastic proteins. Within each type of AFP, the antiserum was more reactive with the protein against which it was raised.

Immunodetection of AFPs in NA and CA Winter Rye Plants

GLPs, CLPs, and TLPs were detected in NA and CA winter rye tissues using immunoblotting techniques. Anti-GLP antiserum raised against the 32-kD GLP recognized two polypeptides (32 and 35 kD) in the apoplast of both CA and NA winter rye leaves (Fig. 3, C and D). These two polypeptides were more difficult to resolve on the blot than on the stained gel (Fig. 3, A–D). This is probably because the gel shrunk slightly in the buffer used for blotting so that the polypeptides appear closer together on the blot. GLP was detected immunologically in NA crowns and in leaves, where it was more concentrated in the apoplast (Fig. 3C). In NA leaves, the GLP was undetectable after apoplastic proteins were removed by centrifugation (Fig. 3C). GLPs were more prominent in extracts of CA crowns and leaves (Fig. 3D). Most of the protein was detected in the apoplastic extract from leaves, although some protein remained in the leaves following the extraction (Fig. 3D). GLPs were not observed in either NA or CA roots (Fig. 3, C and D).

Anti-CLP antiserum raised against the 35-kD native protein recognized several polypeptides in winter rye total protein samples from leaves, crowns, and roots (Fig. 3, E and F). CLP was observed at low levels in NA leaves, where a 35-kD polypeptide was barely detectable in apoplastic extracts (Fig. 3E). On the other hand, both the 35- and 28-kD CLPs accumulated to high levels in apoplastic extracts of CA leaves (Fig. 3F). Polypeptides of 35 and 30 kD were detected in total protein extracts of CA leaves (Fig. 3F). The signal for the 35-kD CLP was weaker after extraction of the apoplast. In NA crowns, two polypeptides at 72 and 30 kD reacted with anti-CLP antiserum (Fig. 3E). These polypeptides, as well as the 35- and 88-kD polypeptides, were more pronounced in the crowns of CA winter rye (Fig. 3F). In NA roots, only a 72-kD polypeptide was observed, whereas in CA roots, polypeptides at 72 and 30 kD were detected (Fig. 3F).

Anti-TLP antiserum was raised against the 25-kD polypeptide. This polypeptide accumulated in extracts of NA leaves and crowns but mostly in the leaf apoplast (Fig. 3G), because it disappeared after extraction of the apoplastic proteins from the leaf (Fig. 3G). In NA leaves, crowns, and roots, a second polypeptide of 27 kD was detected (Fig. 3G). The 25-kD polypeptide accumulated in CA leaves and crowns and mostly in the leaf apoplast (Fig. 3H). After extraction of apoplastic proteins, this polypeptide was less prominent. A second TLP (16 kD) was also observed in apoplastic extracts of CA leaves (Fig. 3H). In CA roots, anti-TLP antiserum detected only a faint 27-kD band (Fig. 3H).

Immunolocalization of AFPs by Tissue Printing

The tissue-printing technique has been successfully applied to localize cellular and extracellular proteins (Varner and Ye, 1994). With this technique, fresh-cut tissue is pressed on the $CaCl_2$-pretreated nitrocellulose paper, and all soluble and salt-soluble proteins (e.g. cell-wall proteins) are transferred onto the paper (Ye and Varner, 1991; del Campillo, 1992). Tissue printing relies on applying uniform pressure to all tissues. When printing soft tissues with a

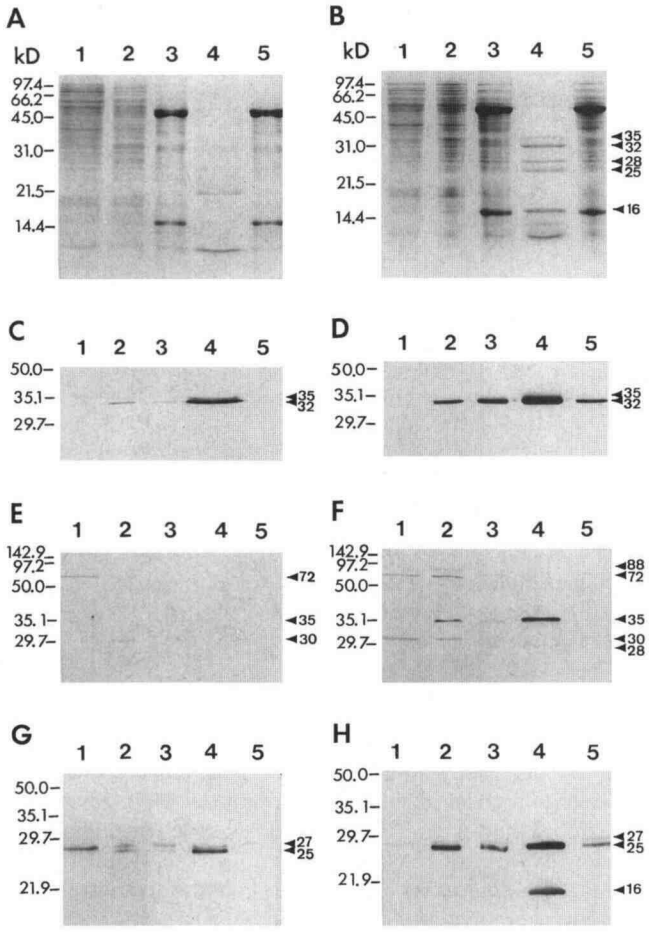

Figure 3. Immunodetection of AFPs in NA (A, C, E, and G) and CA (B, D, F, and H) winter rye tissues. A and B, Coomassie blue-stained SDS-PAGE gels (15% polyacrylamide). C and D, Immunoblots probed with anti-GLP antiserum (dilution 1:500). E and F, Immunoblots probed with anti-CLP antiserum (dilution 1:500). G and H, Immunoblots probed with anti-TLP antiserum (dilution 1:500). Lanes 1, Total soluble proteins from roots (12 μg of protein); lanes 2, total soluble proteins from crowns (12 μg of protein); lanes 3, total soluble proteins from leaves (12 μg of protein); lanes 4, apoplastic proteins from leaves (1.5 μg of protein), NA extract in A, C, E, and G was concentrated prior to loading; lanes 5, leaf total soluble proteins extracted after apoplast was removed by centrifugation (12 μg of protein). Bio-Rad molecular mass markers are indicated on the right in A and B, and Bio-Rad prestained molecular mass markers are marked on the left in C to H. Numbers on the right refer to the molecular masses of the rye polypeptides.

high water content, such as winter rye roots, uniform pressure is difficult to attain, which results in lower image resolution. Because the tissue prints were diffuse, we were unable to determine the precise spatial distribution of proteins at the subcellular level. Therefore, we have limited our interpretation of the immunolocalization results to the cellular level.

Winter rye AFPs were immunolocalized in leaves, crowns, and roots by tissue printing. As shown in Figure 4A, a typical rye leaf has large intercellular spaces between leaf mesophyll cells. Tissue prints of rye leaves immunostained in the absence of primary antibody showed that there was no immunoreaction by the secondary antibody alone because only the background staining caused by Chl was observed (Fig. 5). Tissue prints stained with amido black to show total proteins revealed no staining in the intercellular spaces of NA or CA leaves (Fig. 6, A and D). However, there was a pronounced difference in the localization of AFPs between NA and CA leaves. GLPs, CLPs, and TLPs were all barely detectable in NA leaves (Figs. 6G and 7, A and G) because only weak signals were observed in the mestome sheath encircling the vascular bundles. In CA leaves, GLPs were mostly deposited in cells surrounding the intercellular spaces in the mesophyll, although they were also present in the epidermis (Fig. 6J). CLPs were located mostly in the epidermis of CA leaves, but a high signal was also detected in mesophyll cells lining the intercellular spaces (Fig. 7D). TLPs were present in the epidermis of CA rye leaves but were not detected near the intercellular spaces (Fig. 7J).

Winter rye crowns have a very complicated anatomy, as shown in Figure 4, C and D. GLPs, CLPs, and TLPs all accumulated to a greater extent in the cortex and epidermis of CA crowns (Figs. 6K and 7, E and K) than in NA crowns (Figs. 6H and 7, B and H). The accumulation of TLP in CA crowns was weaker than expected from the results obtained in western analysis of crown total proteins (Fig. 3H, lane 2). However, tissue from the entire crown was ground for total soluble protein extraction and western analysis, whereas proteins localized by tissue printing were present at much lower concentrations on the surface of tissue section. GLPs and CLPs were also clearly localized in the adaxial epidermis of the leaf sheath enveloping the CA crown (Figs. 6K and 7E).

A cross-section of a winter rye root is presented in Figure 4B to show the overall root anatomy. A detailed description of the effect of cold acclimation on root anatomy was reported by Griffith and McIntyre (1990). GLPs did not exhibit any specific localization in either NA or CA roots (Fig. 6, I and L). In contrast, CLPs were localized in the endodermis and the vascular tissue of NA roots (Fig. 7C), whereas they accumulated to a higher degree in the epidermis of CA roots (Fig. 7F). TLPs were detected in the epidermis of NA roots (Fig. 7I) and weakly in CA roots (Fig. 7L).

DISCUSSION

Apoplastic versus Intracellular Location of AFPs

Winter rye has been shown to accumulate proteins in the leaf apoplast during cold acclimation (Griffith et al., 1992; Marentes et al., 1993; Hon et al., 1994). These apoplastic proteins, which have the ability to modify ice crystal growth, were identified as GLPs, CLPs, and TLPs using amino-terminal amino acid sequence comparisons, immuno-cross-reactions, and enzyme activity assays (Hon et al., 1995). The apoplast comprises xylem tracheids and vessels, cell walls, and intercellular spaces. Proteins extracted from the apoplast are soluble and/or loosely bound to cell walls or the outer surface of the plasma membrane (Li et al., 1989; Brune et al., 1994).

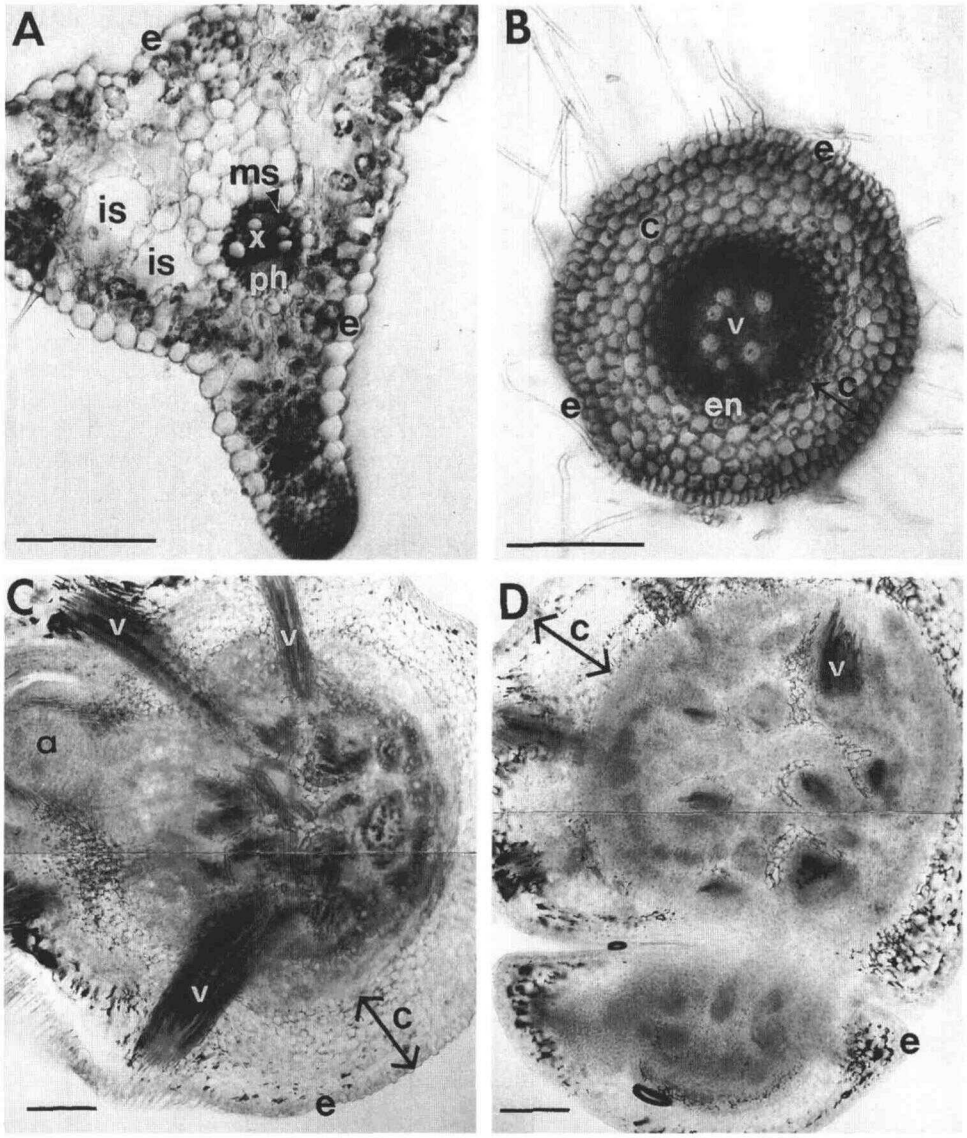

Figure 4. Anatomy of winter rye leaves, roots, and crowns. A, Transverse section taken from mid-length region of a fully expanded NA winter rye leaf showing the vascular bundle of the mid-vein. Two bundle sheaths are present, the inner mestome sheath having smaller cells than the outer parenchymal sheath. Large intercellular spaces are present in the mesophyll. B, Transection of a CA winter rye root taken from 1 to 1.5 cm from the root tip shows the vascular cylinder encircled by the endodermis, cortex, and epidermis. C, Transverse section from a CA winter rye crown cut near the root-shoot junction shows an adventitious root and the vascular bundle leading toward the roots. D, Transverse section of the rye crown sectioned at a greater distance from the root-shoot junction. Sections were stained with phloroglucinol hydrochloride, which stains lignin. Observed tissue types were denoted as follows: a, adventitious root; c, cortex; e, epidermis; en, endodermis; is, intercellular space; ms, mestome sheath; ph, phloem; v, vascular tissue; x, xylem. Scale bar = 200 μm.

Extraction of apoplastic proteins from CA leaves decreased immunologically detectable AFPs in total protein samples of the remaining leaf material (Fig. 3, D, F, and H), which indicates that most AFPs in winter rye are secreted proteins. On the other hand, removal of apoplastic proteins from CA leaves did not significantly decrease the antifreeze activity of the total soluble protein fraction (Fig. 1F), which suggests that all of the AFPs were not extracted by vacuum infiltration followed by centrifugation. Therefore, some AFPs may be more tightly bound to the cell walls, localized in areas that are difficult to extract, or located intracellularly. As a result, we examined the localization of each class of AFP in detail.

A 35-kD GLP was removed from NA and CA rye leaves by apoplastic extraction (Fig. 3, C and D), indicating that it is a secreted protein. A different result was obtained with the 32-kD GLP. This polypeptide accumulated in the apoplast of both NA and CA leaves (Fig. 3, C and D) but was not completely removed by the apoplastic extraction procedure. The possibility that the 32-kD polypeptide also

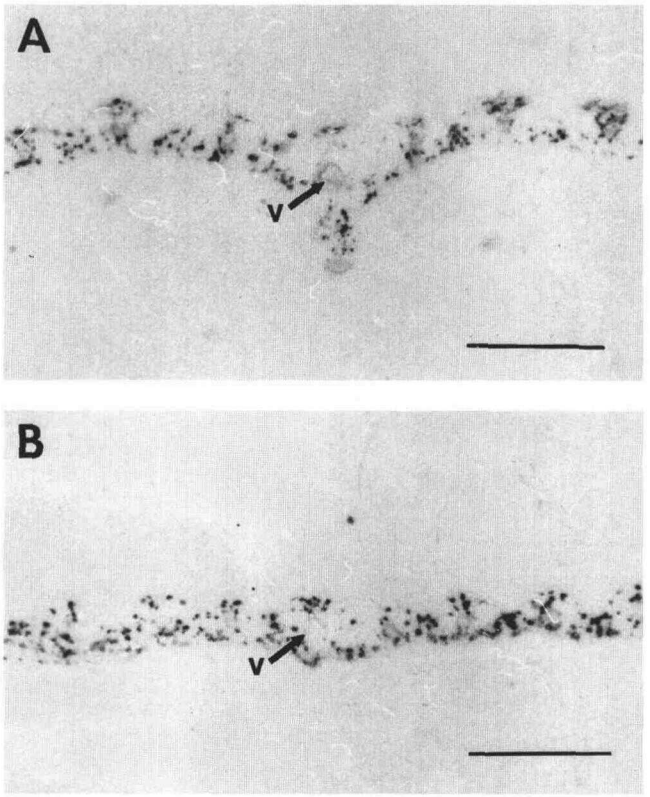

Figure 5. Control tissue prints of cross-sections of an NA (A) and a CA (B) winter rye leaf. The prints were processed for immunolocalization, but the primary antibody was replaced with blocking solution. Tissue prints were incubated in the secondary antibody, followed by visualization by NBT and BCIP. Only background staining due to Chl was visible. v, Location of the vascular bundle of the mid-vein. Scale bar = 500 μm.

accumulated in an intracellular pool was tested using leaf mesophyll protoplasts prepared from NA and CA winter rye leaves (data not shown). No GLP was detected immunologically in either protoplast preparation, a result that supports the conclusion that GLPs accumulate only in the apoplast in winter rye leaves.

In winter rye tissues, the CLP antiserum recognized polypeptides of 88, 72, 35, 30, and 28 kD, which may indicate the production of different isoforms of chitinase in winter rye. In fact, three different chitinases (33 kD, pI = 9.7; 26 kD, pI = 10; 26 kD, pI >10) have been purified from rye seeds (Yamagami and Funatsu, 1993), which shows that different isoforms are produced by this plant. Different isoforms of chitinases have also been reported in other plant species, and the general conclusion in dicots is that class I basic chitinases are localized in vacuoles, whereas other acidic chitinases are located apoplastically (Collinge et al., 1993). Another explanation arises from the multidomain structure of CLPs, which are composed of different combinations of catalytic and chitin-binding domains (Raikhel et al., 1993; Beintema, 1994). The 35-kD CLP from CA winter rye contains a chitin-binding domain because it was purified by affinity chromatography with colloidal chitin. The anti-CLP antiserum may recognize this domain, and therefore the antiserum may also recognize other proteins, such as lectins, that contain one or more chitin-binding domains without the catalytic domain.

A 35-kD CLP detected in apoplastic extracts of NA and CA leaves was almost entirely removed by extracting the leaf apoplast, and a 28-kD CLP was found only in the apoplast of CA winter rye leaves (Fig. 3, E and F). These CLPs are considered to be secreted proteins. In contrast, a 30-kD CLP was detected in CA leaf, crown, and root extracts but not in the leaf apoplast (Fig. 3, E and F). Moreover, the intensity of the polypeptide was similar before and after removal of apoplastic proteins from the leaves, thus suggesting that it was located within the cell.

Although the 16-kD TLP accumulated only in the apoplast of CA rye leaves, the 25-kD TLP was not completely removed from the leaves by apoplastic extraction (Fig. 3H). A third, 27-kD TLP was detected in NA leaves, crowns, and roots and in the leaf apoplast but was barely detectable in CA tissues (Fig. 3, G and H). It is likely that 27-kD TLP is an intracellular form that decreases during cold acclimation. In experiments using soluble proteins from NA and CA mesophyll protoplasts, we detected a strong signal for the 27-kD TLP in NA extracts and a much weaker signal in CA extracts (data not shown). These results indicate that there are both intracellular and apoplastic pools of TLPs in winter rye leaves.

In summary, immunoblotting with anti-AFP antisera revealed that the 35- and 32-kD GLPs, the 35- and 28-kD CLPs, and the 25- and 16-kD TLPs are localized in the apoplast of winter rye tissues. These proteins have all been shown to exhibit antifreeze activity (Hon et al., 1994) and to contribute to the freezing tolerance of CA winter rye leaves (Marentes et al., 1993). On the other hand, the 88-, 72-, and 30-kD CLPs and the 27-kD TLP are thought to be localized intracellularly and their role is not known.

Tissue-Specific Localization of AFPs

GLPs, CLPs, and TLPs all exhibited different spatial distributions within NA and CA winter rye leaves, which may indicate that different isoforms are produced in plants grown at different temperatures. In tissue prints of NA leaves, GLPs, CLPs, and TLPs were weakly localized in the mestome sheath surrounding the vascular bundle (Figs. 6G and 7, A and G). In contrast, all three classes of AFPs were localized in the epidermis of CA leaves, and GLPs and CLPs lined the intercellular spaces (Figs. 6J and 7D). Although the distribution of GLPs, CLPs, and TLPs was similar in the epidermis and cortex of both NA and CA crowns, CA crowns accumulated GLPs and CLPs to a greater extent in the adaxial epidermis of the surrounding leaf sheath (Figs. 6, H and K, and 7, B, E, H, and K). In the roots of NA and CA rye, GLPs and TLPs had similar distributions (Figs. 6, I and L, and 7, I and L), but there was a clear difference in localization of the CLPs. In CA roots, CLPs were localized in the epidermis, whereas CLPs appeared in the endodermis and vascular tissues of NA roots (Fig. 7, C and F).

Even though GLPs, CLPs, and TLPs were present in NA tissues, these proteins did not exhibit antifreeze activity

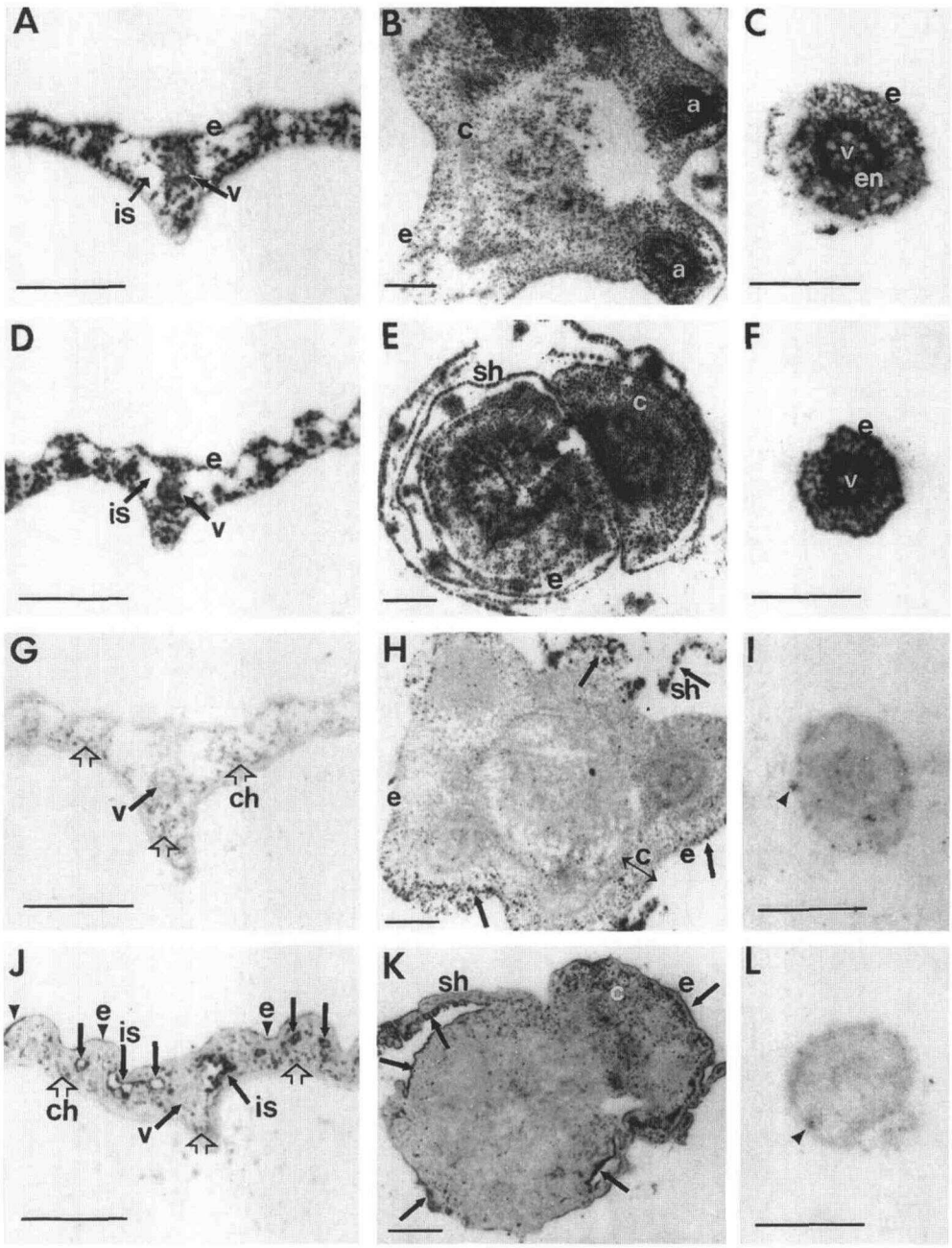

Figure 6. Immunolocalization of AFPs in winter rye leaves, crowns, and roots. Tissue prints of cross-sections of NA leaves (A and G), crowns (B and H), and roots (C and I) and CA leaves (D and J), crowns (E and K), and roots (F and L). Leaves were sectioned from the mid-length region, and the figures show the mid-vein of a mature leaf. Crowns were cross-sectioned near the root-shoot junction (B and H) or from the higher level (E and K). Roots were sectioned 1 to 1.5 cm from the root tip. Tissue prints (A–F) were stained for total protein with amido black. Tissue prints (G–L) were probed with the anti-GLP antiserum (dilution 1:500). a, Adventitious root; c, cortex; ch, Chl; e, epidermis; en, endodermis; is, intercellular space; sh, sheath; v, vascular tissue. The color visible in the leaf mesophyll (designated with open arrows) is not a positive immunoreaction but is stained instead by Chl. On the original prints the chloroplasts stain green, whereas a positive immunological signal (solid arrows and arrowheads) stains purple. Magnification bar = 500 μm.

(Fig. 1). This observation further supports our hypothesis that different isoforms of GLPs, CLPs, and TLPs with antifreeze activity are produced at low temperatures. PR proteins have been reported to be developmentally regulated in healthy plants (Bol et al., 1990; Collinge et al., 1993; Stintzi et al., 1993), and therefore the production of GLPs, CLPs, and TLPs in NA tissues may represent either a particular stage of tissue development or constitutive accumulation of a low level of PR proteins.

Glucanases and chitinases have been localized in other plant species. Ethylene-induced chitinase and β-1,3-glucanase are present in abaxial epidermal cells and in paren-

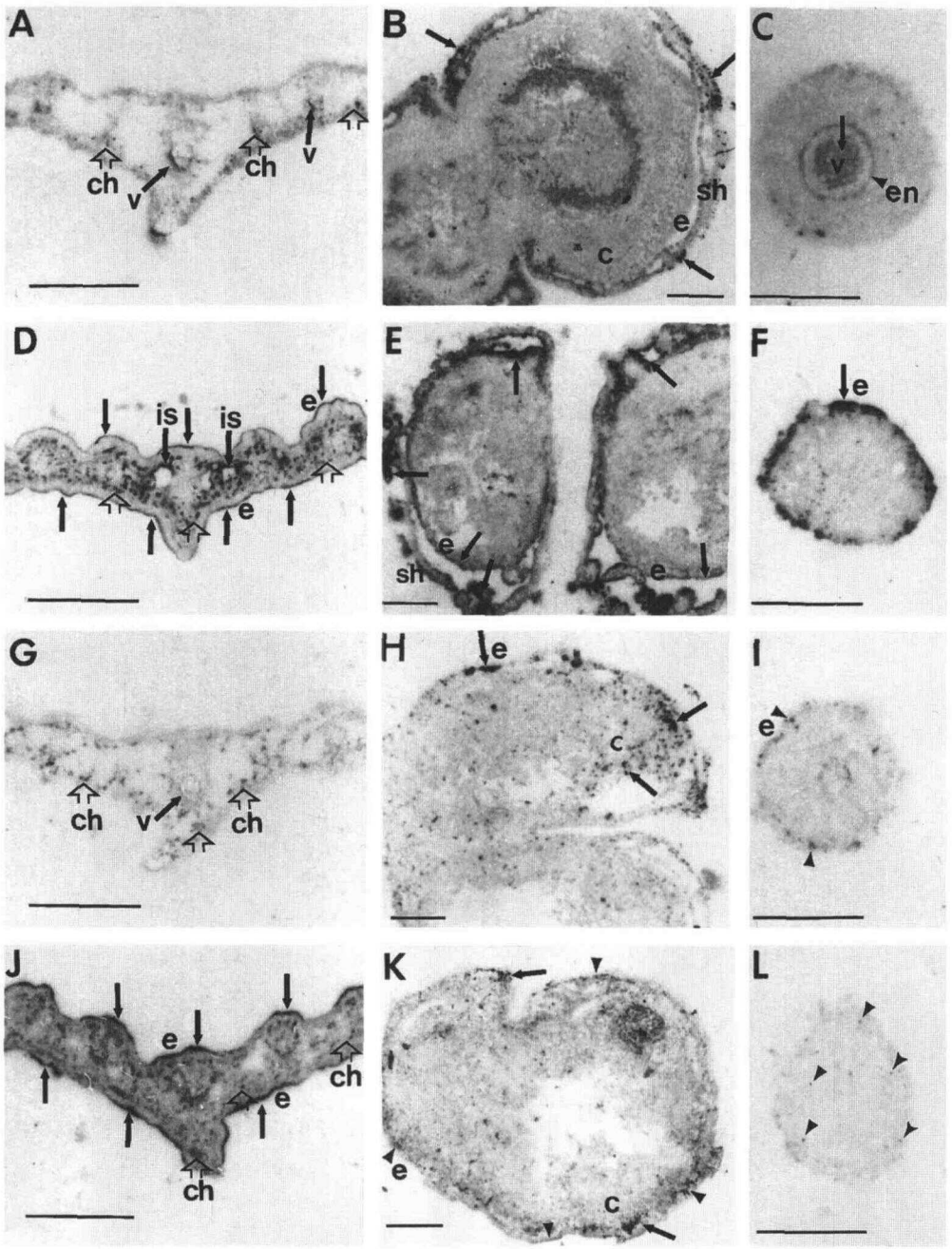

Figure 7. Immunolocalization of AFPs in winter rye leaves, crowns, and roots. Tissue prints of NA leaves (A and G), crowns (B and H), and roots (C and I) and CA leaves (D and J), crowns (E and K), and roots (F and L). Tissue prints were probed with anti-CLP antiserum (dilution 1:500) (A–F) and with anti-TLP antiserum (dilution 1:500) (G–L). c, Cortex; ch, Chl; e, epidermis; en, endodermis; is, intercellular space; sh, sheath; v, vascular tissue. Black spots in the mesophyll (designated with open arrows) are not a positive reaction of the antiserum (filled arrows and arrowheads) but are instead stained by Chl. Magnification bar = 500 μm.

chymal cells adjacent to vascular strands in bean leaves (Mauch et al., 1992). β-1,3-Glucanase is also distributed along the surface layer of the cell wall, lining all of the airspaces (Mauch et al., 1992). In tomato leaves, pathogen-induced β-1,3-glucanase and chitinase accumulate near the stomata in the abaxial epidermal layer (Wubben et al., 1993). The distributions of pathogen-induced and low-temperature-induced GLPs, CLPs, and TLPs are similar and may reflect the common pathways for the penetration and propagation of pathogens and ice. Both pathogens and extracellular ice may enter leaves through the stomata, and both ice and pathogens may propagate through the vascular system of a plant. Because winter cereals are more resistant to fungal diseases after cold acclimation (Tronsmo, 1984, 1985; Tronsmo et al., 1993), it is possible that winter rye AFPs may also have a role in pathogen resistance.

Possible Roles of AFPs

There are two possible roles for AFPs in the apoplast of CA winter rye plants. First, these AFPs are associated with the epidermis and with cells lining intercellular spaces. If the AFPs are not released from the cells, then the AFPs may function as barriers to ice propagation into the plant or into individual cells. For example, the epidermis provides a barrier against the likelihood of secondary ice nucleation originating from ice formed on the surface of the leaf. In CA winter rye, epidermal modifications include reduced leaf surface area, fewer stomates per leaf area (Huner et al., 1981), and a dramatic thickening of the cell wall (Griffith and Brown, 1982), cuticular layer, and epicuticular waxes (Griffith et al., 1985). We have now shown that GLPs, CLPs, and TLPs with antifreeze activity all accumulate in the epidermis of CA leaves as well. Epidermal AFPs, coupled with cutinized cell walls and increased epicuticular waxes, may provide barriers for both ice propagation and penetration of pathogens from outside of the plant.

Barriers to ice propagation may also exist within the plant itself. In winter rye leaves frozen under laboratory conditions, ice crystals form in xylem vessels and in intercellular spaces around vascular bundles, below the epidermis and between adjoining mesophyll cells (Pearce, 1988). By comparison, in field-frozen wheat, extracellular ice is located mainly in intercellular spaces below the epidermis and in substomatal cavities (Pearce and Ashworth, 1992). The distribution of AFPs around the intercellular spaces of CA winter rye leaves is consistent with the location of extracellular ice. We hypothesize that GLPs and CLPs associated with cells around vascular bundles may be important in slowing secondary ice nucleation from xylem. AFPs located in cells surrounding intercellular spaces may also prevent inoculative freezing of cells. A similar conclusion was reached by Jian et al. (1987), who proposed that glycoproteins localized at the free cell surface bordering intercellular spaces in CA winter wheat leaves could form a protective barrier to prevent ice nuclei from entering cells.

Both insects and fish may use AFPs to prevent secondary ice nucleation. In insects, AFPs are associated with the epidermal layer located just beneath the cuticle (Duman et al., 1993). In fish, AFPs are present in the skin of winter flounder, shorthorn sculpin, and cunner and form an effective barrier against ice propagation (Schneppenheim and Theede, 1982; Valerio et al., 1990, 1992).

A second possible role of rye AFPs is to inhibit the recrystallization of ice. In this case, the AFPs may not be tightly bound to the cells so that they are free to bind to the surfaces of extracellular ice crystals. One example of the role of AFPs in the inhibition of recrystallization may occur within the crown. The survival of the crown is essential for winter survival of crown (Chen et al., 1983). However, the crown does not freeze uniformly because of the highly heterogeneous structure of the tissue (Olien, 1967; Millard et al., 1995), the accumulation of carbohydrates (Olien and Lester, 1985), and modifications of the xylem in the root-shoot junction that slow the propagation of ice (Aloni and Griffith, 1991; Zámečník et al., 1994). Nonuniform freezing may lead to the production of larger ice crystals, which could be inhibited by AFPs. Our results show that all three classes of AFPs were localized in CA rye crowns (Figs. 6 and 7) and antifreeze activity was present in the total soluble protein extract of the crown (Fig. 1). Thus, the AFPs may modify the freezing process to maintain small crystal size in the crowns of CA winter rye plants.

Only a low level of antifreeze activity was observed in CA winter rye roots (Fig. 1J), which is correlated with their low degree of freezing tolerance (−7 to −8°C; Griffith and McIntyre, 1990). However, root survival may not be critical to an overwintering plant, because the roots may continue to function as conduits for water and nutrient transport as long as the xylem vessels are not embolized (Aloni and Griffith, 1991). Only the 72- and 30-kD CLPs were detected immunologically in CA rye roots (Fig. 3F), which may indicate that these proteins may have other functions, such as providing a defence against soil-borne pathogens.

In summary, GLPs, CLPs, and TLPs with antifreeze activity are localized in the apoplast of CA winter rye tissues. The distribution of these proteins in the epidermis and around intercellular spaces is correlated with the location of both epiphytic and extracellular ice, leading us to conclude that these proteins do have a function in modifying the growth of ice. Moreover, the GLPs, CLPs, and TLPs present in NA plants lacked antifreeze activity and were generally localized in different cell types, which suggests that the AFPs may be different isoforms of PR proteins.

ACKNOWLEDGMENTS

We thank Dr. C.A. Peterson, D. Enstone, Dr. W. Diehl-Jones, K. Alho, P. Saarenmaa, M. Stevens, and X.-M. Yu for technical assistance. We are grateful for the gifts of Musketeer rye seeds provided by Dr. G. McLeod (Agriculture Canada, Swift Current, SK, Canada), anti-tobacco basic β-1,3-glucanase IgG provided by Dr. Frederick Meins, Jr. (Friedrich Miescher-Institut, Basel, Switzerland), anti-tobacco TLP antiserum provided by Dr. W.S. Pierpoint (Agricultural and Food Research Council Institute of Arable Crops Research, Rothamsted Experimental Station, Harpenden, Hertfordshire, UK), and anti-tobacco endochitinase antiserum provided by Dr. Michel Legrand (Centre National de la Recherche Scientifique, Institut de Biologie Moleculaire des Plantes, Strasbourg, France).

Received August 18, 1995; accepted December 6, 1995.
Copyright Clearance Center: 0032-0889/96/110/0845/13.

LITERATURE CITED

Aloni R, Griffith M (1991) Functional xylem anatomy in root-shoot junctions of six cereal species. Planta **184:** 123–129

Beintema JJ (1994) Structural features of plant chitinases and chitin-binding proteins. FEBS Lett **350:** 159–163

Bol JF, Linthorst HJM, Cornelissen BJC (1990) Plant pathogenesis-related proteins induced by virus infection. Annu Rev Phytopathol **28:** 113–138

Boller T, Gehri A, Mauch F, Vögeli U (1983) Chitinase in bean leaves: induction by ethylene, purification, properties, and possible function. Planta **157:** 22–31

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 341–374

Brune A, Urbach W, Dietz K-J (1994) Zinc stress induces changes in apoplasmic protein content and polypeptide composition of barley primary leaves. J Exp Bot **45:** 1189–1196

Brush RA, Griffith M, Mlynarz A (1994) Characterization and quantification of intrinsic ice nucleators in winter rye (*Secale cereale*) leaves. Plant Physiol **104:** 725–735

Carr JP, Klessig DF (1989) The pathogenesis-related proteins of plants. *In* JK Setlow, ed, Genetic Engineering, Principles and Methods, II. Plenum Press, New York, pp 65–109

Chen TH-H, Gusta L, Fowler DB (1983) Freezing injury and root development in winter cereals. Plant Physiol **73:** 773–777

Collinge DB, Kragh KM, Mikkelsen JD, Nielsen KK, Rasmussen U, Vad K (1993) Plant chitinases. Plant J **3:** 31–40

del Campillo E (1992) Induction, accumulation, localization, and transport studies by tissue printing. *In* PD Reid, RF Pont-Lezica, eds, Tissue Printing: Tools for the Study of Anatomy, Histochemistry, and Gene Expression. Academic Press, San Diego, CA, pp 41–58

DeVries AL (1986) Antifreeze glycopeptides and peptides: interactions with ice and water. Methods Enzymol **127:** 293–303

Duman JG (1994) Purification and characterization of a thermal hysteresis protein from a plant, the bittersweet nightshade *Solanum dulcamara*. Biochim Biophys Acta **1206:** 129–153

Duman JG, Wu DW, Olsen TM, Urrutia M, Tursman D (1993) Thermal-hysteresis proteins. Adv Low-Temp Biol **2:** 131–182

Ernst D, Schraudner M, Langebartels C, Sanderman H Jr (1992) Ozone-induced changes of mRNA levels of β-1,3-glucanase, chitinase and "pathogenesis-related" protein 1B in tobacco plants. Plant Mol Biol **20:** 673–682

Esaka M, Touota A, Hayakawa H (1994) Secretion of basic and acidic chitinases from salt-adapted and -unadapted winged bean cells. Physiol Plant **92:** 90–96

Gatschet MJ, Taliaferro CM, Porter DR, Anderson MP, Anderson JA, Jackson KW (1996) A cold-regulated protein from bermudagrass crowns is a chitinase. Crop Sci (in press)

Ghosh S, Gepstein S, Glick BR, Heikkila JJ, Dumbroff EB (1989) Thermal regulation of phospho*enol*pyruvate carboxylase and ribulose-1,5-bisphosphate carboxylase in C_3 and C_4 plants native to hot and temperate climates. Plant Physiol **90:** 1298–1304

Griffith M, Ala P, Yang DSC, Hon W-C, Moffatt BA (1992) Antifreeze protein produced endogenously in winter rye leaves. Plant Physiol **100:** 593–596

Griffith M, Antikainen M (1996) Extracellular ice formation in plants. Adv Low-Temp Biol **3:** (in press)

Griffith M, Brown GN (1982) Cell wall deposits in winter rye *Secale cereale* L. "Puma" during cold acclimation. Bot Gaz **143:** 486–490

Griffith M, Huner NPA, Espelie KE, Kolattukudy PE (1985) Lipid polymers accumulate in the epidermis and mestome sheath cell walls during low temperature development of winter rye leaves. Protoplasma **125:** 53–64

Griffith M, McIntyre HCH (1990) The effect of photoperiod and temperature on growth and frost resistance of winter rye root system. Physiol Plant **79:** 519–525

Guy C (1990) Cold acclimation and freezing stress tolerance: role of protein metabolism. Annu Rev Plant Physiol Plant Mol Biol **41:** 187–223

Hon W-C, Griffith M, Chong P, Yang DSC (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, Kwok YC, Yang DSC (1995) Antifreeze proteins in winter rye are similar to pathogenesis-related proteins. Plant Physiol **109:** 879–889

Huner NPA, Macdowall FDH (1976) Chloroplastic proteins of wheat and rye grown at cold-hardening temperatures. Can J Biochem **54:** 848–853

Huner NPA, Palta JP, Li PH, Carter JV (1981) Anatomical changes in leaves of Puma rye in response to growth at cold-hardening temperatures. Bot Gaz **142:** 55–62

Huynh QK, Hironaka CM, Levine EB, Smith CE, Bormeyer JR, Shah DM (1992) Antifungal proteins from plants: purification, molecular cloning, and antifungal properties of chitinases from maize seed. J Biol Chem **267:** 6635–6640

Jacobsen S, Hauschild MZ, Rasmussen U (1992) Induction by chromium ions of chitinases and polyamines in barley (*Hordeum vulgare* L.) and rape (*Brassica napus* L. ssp. *oleifera*). Plant Sci **84:** 119–128

Jian LC, Sun LH, Sun DL (1987) Glycoproteins at the cell surface in cold hardy and cold tender wheat (*Triticum aestivum* L.). *In* PH Li, ed, Plant Cold Hardiness. Alan R Liss, New York, pp 59–66

Kärenlampi SO, Airaksinen K, Miettinen ATE, Kokko HI, Holopainen JK, Kärenlampi L, Karjalainen RO (1994) Pathogenesis-related proteins in ozone-exposed Norway spruce [*Picea abies* (Karst) L.]. New Phytol **126:** 81–89

Keefe D, Hinz U, Meins F Jr (1990) The effect of ethylene on the cell-type-specific and intracellular localization of β-1,3-glucanase and chitinase in tobacco leaves. Planta **182:** 43–51

Knight CA, Duman JG (1986) Inhibition of recrystallization of ice by insect thermal hysteresis proteins: a possible cryoprotective role. Cryobiology **23:** 256–262

Kurkela S, Franck M, Heino P, Lång V, Palva ET (1988) Cold induced gene expression in *Arabidopsis thaliana* L. Plant Cell Rep **7:** 495–498

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Legrand M, Kauffman S, Geoffroy P, Fritig B (1987) Biological function of "pathogenesis-related" proteins: four tobacco pathogenesis-related proteins are chitinases. Proc Natl Acad Sci USA **84:** 6750–6754

Li Z-C, McClure JW, Hagerman AE (1989) Soluble and bound apoplastic activity for peroxidase, β-D-glucosidase, malate dehydrogenase, and nonspecific arylesterase, in barley (*Hordeum vulgare* L.) and oat (*Avena sativa* L.) primary leaves. Plant Physiol **90:** 185–190

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem **193:** 265–275

Marentes E, Griffith M, Mlynarz A, Brush RA (1993) Proteins accumulate in the apoplast of winter rye leaves during cold acclimation. Physiol Plant **87:** 499–507

Margis-Pinheiro M, Marivet J, Burkard G (1994) Bean class IV chitinase gene: structure, developmental expression and induction by heat stress. Plant Sci **98:** 163–173

Mauch F, Meehl JB, Staehelin LA (1992) Ethylene-induced chitinase and β-1,3-glucanase accumulate specifically in the lower epidermis and along vascular strands of bean leaves. Planta **186:** 367–375

Millard MM, Veisz OB, Krizek DT, Line M (1995) Magnetic resonance imaging (MRI) of water during cold acclimation and freezing in winter wheat. Plant Cell Environ **18:** 535–544

Olien CR (1967) Freezing stresses and survival. Annu Rev Plant Physiol **18:** 387–408

Olien CR, Lester GE (1985) Freeze-induced changes in soluble carbohydrates of rye. Crop Sci **25:** 288–290

Palva ET (1994) Gene expression under low temperature stress. *In* AS Basra, ed, Stress-Induced Gene Expression in Plants. Harwood Academic, Chur, Switzerland, pp 103–130

Pearce RS (1988) Extracellular ice and cell shape in frost-stressed cereal leaves: a low-temperature scanning-electron-microscopy study. Planta **175:** 313–324

Pearce RS, Ashworth EN (1992) Cell shape and localization of ice in leaves of overwintering wheat during frost stress in the field. Planta **188:** 324–331

Pierpoint WS, Gordon-Weeks R, Jackson PJ (1992) The occurrence of the thaumatin-like pathogenesis-related protein, PR-5, in intercellular fluids from *Nicotiana* species and from an interspecific *Nicotiana* hybrid. Physiol Mol Plant Pathol **41:** 1–10

Raikhel NV, Lee H-I, Broekaert WF (1993) Structure and function of chitin-binding proteins. Annu Rev Plant Physiol Plant Mol Biol **44:** 591–615

Schneppenheim R, Theede H (1982) Freezing-point depressing polypeptides and glycopeptides from Arctic-boreal and Antarctic fish. Polar Biol **1:** 115–123

Stintzi A, Heitz T, Prasad V, Wiedemann-Merdinoglu S, Kauffmann S, Geoffroy P, Legrand M, Fritig B (1993) Plant "patho-

genesis-related" proteins and their role in defense against pathogens. Biochimie **75:** 687–706

Tronsmo AM (1984) Predisposing effects of low temperature on resistance to winter stress factors in grasses. Acta Agric Scand **34:** 210–220

Tronsmo AM (1985) Induced resistance to biotic stress factors in grasses by frost hardening. *In* Å Kaurin, O Junttila, eds, Plant Production in the North. Norwegian University Press, Tromsø, Norway, pp 127–133

Tronsmo AM, Gregersen P, Hjeljord L, Sandal T, Bryngelsson T, Collinge DB (1993) Cold-induced disease resistance. *In* B Fritig, M Legrand, eds, Mechanisms of Plant Defense Responses. Kluwer Academic, Dordrecht, The Netherlands, p 369

Valerio PF, Kao MH, Fletcher GL (1990) Thermal hysteresis activity in the skin of the cunner, *Tautogolabrus adspersus*. Can J Zool **68:** 1065–1067

Valerio PF, Kao MH, Fletcher GL (1992) Fish skin: an effective barrier to ice propagation. J Exp Biol **164:** 135–151

Varner JE, Ye Z (1994) Tissue printing. FASEB J **8:** 378–384

Wubben JP, Eijkelboom CA, De Wit PJGM (1993) Accumulation of pathogenesis-related proteins in the epidermis of tomato leaves infected by *Cladosporium fulvum*. Neth J Plant Pathol **99:** (3 Suppl) 231–239

Yalpani N, Enyedi AJ, León J, Raskin I (1994) Ultraviolet light and ozone stimulate accumulation of salicylic acid, pathogenesis-related proteins and virus resistance in tobacco. Planta **193:** 372–376

Yamagami T, Funatsu G (1993) Purification and some properties of three chitinases from the seeds of rye (*Secale cereale*). Biosci Biotechnol Biochem **57:** 643–647

Ye Z-H, Varner JE (1991) Tissue-specific expression of cell wall proteins in developing soybean tissues. Plant Cell **3:** 23–37

Zámečník J, Bieblová J, Grospietch M (1994) Safety zone as a barrier to root-shoot ice propagation. Plant Soil **167:** 149–155

Zhang Y-Y, Punja ZK (1994) Induction and characterization of chitinase isoforms in cucumber (*Cucumis sativus* L.): effect of elicitors, wounding and pathogen inoculation. Plant Sci **99:** 141–150

Zhu B, Chen THH, Li PH (1995) Expression of three osmotin-like protein genes in response to osmotic stress and fungal infection in potato. Plant Mol Biol **28:** 17–26