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Antifreeze Proteins in Winter Rye Leaves Form Oligomeric Complexes[1]

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Antifreeze proteins (AFPs) similar to three pathogenesis-related proteins, a glucanase-like protein (GLP), a chitinase-like protein (CLP), and a thaumatin-like protein (TLP), accumulate during cold acclimation in winter rye (*Secale cereale*) leaves, where they are thought to modify the growth of intercellular ice during freezing. The objective of this study was to characterize the rye AFPs in their native forms, and our results show that these proteins form oligomeric complexes in vivo. Nine proteins were separated by native-polyacrylamide gel electrophoresis from apoplastic extracts of cold-acclimated winter rye leaves. Seven of these proteins exhibited multiple polypeptides when denatured and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After isolation of the individual proteins, six were shown by immunoblotting to contain various combinations of GLP, CLP, and TLP in addition to other unidentified proteins. Antisera produced against individual cold-induced winter rye GLP, CLP, and TLP all dramatically inhibited glucanase activity in apoplastic extracts from cold-acclimated winter rye leaves, and each antiserum precipitated all three proteins. These results indicate that each of the polypeptides may be exposed on the surface of the protein complexes. By forming oligomeric complexes, AFPs may form larger surfaces to interact with ice, or they may simply increase the mass of the protein bound to ice. In either case, the complexes of AFPs may inhibit ice growth and recrystallization more effectively than the individual polypeptides.

Winter rye (*Secale cereale*) is an overwintering plant that survives freezing to temperatures less than −20°C by forming ice in intercellular spaces (Pearce, 1988; Brush et al., 1994). As winter rye plants acclimate to low temperatures, they secrete proteins into the leaf apoplast, where ice forms. These apoplastic proteins accumulate to levels of about 0.3 mg protein g^{-1} leaf fresh weight after 7 weeks of cold acclimation, and they decrease dramatically in concentration within a few days if the plants are returned to 20°C to deacclimate (Marentes et al., 1993). Many of these are AFPs, which modify the growth of ice crystals and inhibit the recrystallization of ice when assayed in vitro (Griffith et al., 1992; Hon et al., 1994; Griffith and Antikainen, 1996). Although the AFPs are an important component of winter survival in winter cereals (Chun et al., 1997), we know little about how these proteins function in vivo. The objective of this study was to characterize rye AFPs in their native forms.

When the apoplastic proteins from cold-acclimated winter rye leaves are denatured and separated by SDS-PAGE, six major polypeptides with molecular masses ranging from 16 to 35 kD are present. All six polypeptides exhibit antifreeze activity when assayed individually (Hon et al., 1994). However, these six polypeptides are not unique proteins, as shown by amino-terminal amino acid sequencing, immunoblotting, and assays of enzymatic activity (Hon et al., 1995). Two of the apoplastic polypeptides were identified as GLPs, two were CLPs, and two were TLPs. GLPs, CLPs, and TLPs are also known as pathogenesis-related proteins because they can be induced to accumulate in plants by many plant pathogens, and the presence of pathogenesis-related proteins is positively correlated with disease resistance (Carr and Klessig, 1989; Stintzi et al., 1993). Although β-1,3-endoglucanase and endochitinase activities are present in crude apoplastic extracts of both nonacclimated and cold-acclimated leaves, only apoplastic extracts obtained from cold-acclimated leaves have antifreeze activity (Hon et al., 1995). In fact, a native chitinase purified from cold-acclimated leaves exhibits both antifreeze activity and endochitinase activity (Hon et al., 1995). Because the apoplastic proteins that accumulate at cold temperature in winter rye have both antifreeze and enzymatic activities, these proteins may play dual roles in freezing tolerance and resistance to low-temperature diseases.

Our first attempts at isolating the native AFPs from apoplastic extracts of cold-acclimated winter rye leaves showed that the antifreeze polypeptides coeluted during gel-filtration chromatography (Griffith et al., 1992), which suggested that they may be associated in complexes. To elucidate the possible synergistic role of AFPs in the mechanism of freezing tolerance of the winter rye plant, we characterized winter rye AFPs accumulated during cold acclimation in their native forms using three different approaches: (a) separation of cold-induced apoplastic proteins by native-PAGE and chitin-affinity chromatography; (b) examination of each NP by SDS-PAGE and immunoblotting; and (c) immunoinhibition of glucanase activity and immunoprecipitation of AFPs in cold-acclimated rye apoplastic extracts. Our results indicate that the winter rye AFPs form oligomeric complexes in vivo.

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Abbreviations: AFP, antifreeze protein; CLP, chitinase-like protein; GLP, β-1,3-glucanase-like protein; NP, native protein; TLP, thaumatin-like protein.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Winter rye (*Secale cereale* L. cv Musketeer) seeds were surface-sterilized in a 0.3% sodium hypochlorite solution for 5 min, rinsed with distilled water several times, planted in 15-cm pots of coarse vermiculite, and germinated at 20°C/16°C (day/night) with a 16-h daylength and a PPFD of 300 μmol m^{-2} s^{-1} for 1 week. Nonacclimated plants were grown under the same conditions for an additional 2 weeks. Cold-acclimated plants were transferred to 5°C/2°C (day/night) with an 8-h daylength and a PPFD of 300 μmol m^{-2} s^{-1} for an additional 7 weeks. Nonacclimated plants grown at 20°C/16°C for 3 weeks are similar in physiological age to cold-acclimated plants grown at 5°C/2°C for 7 weeks (Krol et al., 1984; Griffith and McIntyre, 1993). Plants were watered as needed with modified Hoagland solution (Huner and Macdowall, 1976).

Apoplastic Protein Extraction

Apoplastic proteins were extracted by vacuum infiltrating the leaves with extraction buffer containing 20 mM ascorbic acid and 20 mM $CaCl_2$, followed by centrifugation at 900*g* to recover the proteins (Hon et al., 1994). Total protein was measured using the Bradford (1976) method, as modified by Bio-Rad, with BSA as the standard protein. Diluted crude apoplastic extracts were concentrated about 2-fold for cold-acclimated samples and 10-fold for nonacclimated samples, as needed, by ultrafiltration (Centriprep-10, Amicon, Beverly, MA).

Protein Electrophoresis and Purification

Apoplastic proteins extracted from rye leaves were separated with an 8% (w/v) continuous native-PAGE gel using the Mini-Protein II cell and a single-well preparative comb according to the manufacturer's instructions (Bio-Rad). The gel buffer was 30 mM β-Ala and 20 mM lactic acid, pH 3.8. To locate the position of each protein band, a 0.5-cm gel strip was cut from each of the two longitudinal edges of the gel immediately after electrophoresis, stained with 0.1% (w/v) Coomassie brilliant blue R-250 in 40% (v/v) methanol and 10% (v/v) acetic acid (for 10 min), destained with 40% (v/v) methanol and 10% (v/v) acetic acid (for 20 min), and then carefully matched to the remaining gel. Gel pieces corresponding to the individual proteins shown on the two Coomassie blue-stained gel strips were cut from the remaining gel. The gel pieces of each NP were placed in 5-fold-diluted gel buffer and homogenized using a gel nebulizer (Amicon). The homogenized gel slurries were sonicated overnight to allow the proteins to diffuse out of the gel. After centrifugation at 14,500*g* for 10 min, the NPs were recovered from the supernatant and concentrated in one step with Micropure separators and Microcon microconcentrators (Amicon). These procedures were carried out at 4°C. Each of the NPs from the apoplastic extracts was denatured, and the component polypeptides were separated by SDS-PAGE (15% [w/v] acrylamide) and stained with Coomassie brilliant blue R-250 according to the method of Laemmli (1970).

Immunoblotting

Isolated NPs and polypeptides were transferred onto 0.45-μm nitrocellulose membranes (Bio-Rad) using the Mini Trans-Blot cell (Bio-Rad) according to the manufacturer's instructions. A solution of 0.7% (v/v) acetic acid, pH 3.8, was used to transfer NPs, and a buffer composed of 25 mM Tris, 192 mM Gly, and 20% (v/v) methanol, pH 8.3, was used to transfer polypeptides. The blots were probed with the anti-GLP antiserum (dilution, 1:2,000), the anti-CLP antiserum (dilution, 1:2,000), or the anti-TLP antiserum (dilution, 1:10,000) produced against isolated winter rye AFPs similar to GLPs, CLPs, and TLPs, respectively (Antikainen et al., 1996). The immunoreactions were detected by alkaline phosphatase conjugated to goat anti-rabbit IgG (Sigma) with 5-bromo-4-chloro-3-indolyl phosphate-toluidine salt (BioShop, Burlington, Ontario, Canada) and nitroblue tetrazolium (Sigma) as substrates.

Glucanase Activity Assay and Immunoinhibition

Total β-1,3-glucanase (EC 3.2.16) activity was assayed colorimetrically using laminarin (Sigma) as a substrate and dinitrosalicylic reagent to detect the reducing sugars produced, according to the method of Abeles and Forrence (1970), with some modifications. Crude apoplastic extract (50 μL) was added to 50 μL of 1% (w/v) laminarin in the extraction buffer and then incubated at 37°C for 10 min. The reaction was stopped by adding 300 μL of dinitrosalicylic reagent and heating at 95°C for 5 min. The resulting colored solution was cooled to room temperature and diluted 1:10 with distilled, deionized water, and the A_{500} was read using a microplate reader (model EL308, Bio-Tek, Burlington, VT). The blank was a mixture of 50 μL of crude extract, 50 μL of 1% laminarin, and 300 μL of dinitrosalicylic reagent. The specific enzyme activity was defined as the amount of enzyme that produced reducing sugar at a rate of 1 nmol Glc equivalents s^{-1} mg^{-1} protein.

For the experiment involving immunoinhibition of β-1,3-glucanase activity, crude apoplastic extracts from cold-acclimated plants were incubated (apoplastic extract:antiserum, 2:1 [v/v]) with the preimmune serum, anti-GLP antiserum, anti-CLP antiserum, or anti-TLP antiserum at 25°C for 20 min, and then total β-1,3-glucanase activity was assayed as described above.

Immunoprecipitation of AFPs

Crude apoplastic extract from cold-acclimated rye leaves (500 μL) was mixed with 200 μL of protein A-Sepharose CL-4B (Sigma) preswollen in extraction buffer containing 20 mM ascorbic acid and 20 mM $CaCl_2$ to remove apoplastic proteins that bind nonspecifically to the beads. After gentle shaking at room temperature for 4 h and centrifugation at 17,300*g* for 10 min, the beads were discarded. The supernatant was mixed with preimmune serum, anti-GLP antiserum, anti-CLP antiserum, or anti-TLP antiserum (100 μL)

and shaken gently at room temperature for 2 h. Fifty microliters of protein A-Sepharose CL-4B (Sigma) preswollen in extraction buffer containing 20 mM ascorbic acid and 20 mM $CaCl_2$ was added to the extract-antiserum mixture. After the sample was shaken for 2 h at room temperature and centrifuged at 17,300*g* for 5 min, the supernatant was discarded and the pellet was washed five times with extraction buffer to remove unbound proteins. The washed pellet was denatured with SDS reducing buffer (Laemmli, 1970) at 90°C for 5 min and centrifuged at 17,300*g* for 10 min. The supernatant was analyzed by SDS-PAGE and immunoblotting.

Purification of Chitinase

The native rye chitinase was purified from apoplastic extracts of cold-acclimated winter rye leaves by affinity chromatography using colloidal chitin as the column substrate (Huynh et al., 1992; Hon et al., 1995). The purity of chitinase was examined on 8% native-PAGE and 15% SDS-PAGE gels stained with Coomassie brilliant blue R-250, followed by immunoblotting in which antisera produced against cold-induced rye CLP, GLP, and TLP were used as probes.

Antifreeze Activity Assay

Antifreeze activity was assayed qualitatively by examining the morphological characteristics of ice crystals (DeVries, 1986) grown in apoplastic extracts of cold-acclimated rye leaves, in solutions of individual proteins eluted from native gels, and in chitinase fractions purified by chitin-affinity chromatography. The growth of ice crystals in each sample was controlled by a nanoliter osmometer (Clifton Technical Physics, Hartford, NY), and the morphological characteristics of ice crystals were examined using a phase-contrast photomicroscope (model BHT, Olympus). A rating system was developed to quantify and compare the effects of different apoplastic extracts on ice-crystal growth (Chun et al., 1997). The disc-like crystals grown in water were rated 0 because they have no antifreeze activity. Hexagonal discs grown in very low (nanomolar) concentrations of AFPs or in solutions of AFPs with low specific activity were rated 1. Hexagonal columns grown in dilute (micromolar) solutions of AFPs or in solutions of AFPs with moderate specific activity were rated 3. Crystals forming complex hexagonal bipyramids in high concentrations (≥100 μM) of AFPs or in solutions of AFP with high specific activity were rated 5.

RESULTS

Separation of Apoplastic Proteins

Proteins present in the apoplastic extracts from the leaves of nonacclimated plants and plants cold acclimated for 7 weeks were quantified and examined under nondenaturing conditions. The concentration of extractable apoplastic proteins was about 10-fold higher in cold-acclimated apoplastic extracts (0.43 ± 0.05 mg protein mL^{-1}; $n = 5$) than in nonacclimated apoplastic extracts (0.04 ± 0.01 mg mL^{-1}; $n = 4$). We first tried to separate the native apoplastic proteins on a sizing column (1 × 50 cm; Bio-Gel P-100, Bio-Rad) eluted with 30 mM β-Ala and 20 mM lactic acid, pH 3.8, at a rate of 70 μL min^{-1}. Although BSA (67 kD), ovalbumin (43 kD), chymotrypsinogen A (25 kD), and Cyt *c* (12.4 kD) were easily separated on the column, the native apoplastic proteins from cold-acclimated rye leaves yielded only one peak with a trailing shoulder (data not shown). According to the standard curve, these proteins had molecular masses well below 12 kD, which indicated that the proteins were eluted from the column at a slower rate than predicted by their apparent sizes on SDS-PAGE. A similar result was reported earlier when a sizing column packed with Sephacryl 200 was used (Griffith et al., 1992).

In contrast to the results with the open columns, good resolution and consistent separations were obtained when a continuous native-polyacrylamide (8% [w/v]) gel system was used at pH 3.8. As shown in Figure 1, nine NPs were identified in apoplastic extracts from cold-acclimated rye leaves, whereas only six NPs were present in apoplastic extracts from nonacclimated leaves when equal amounts of apoplastic proteins were separated by native-PAGE. Although NP2, NP4, NP5, NP6, NP8, and NP9 were present in both cold-acclimated and nonacclimated leaves, NP1, NP3, and NP7 were detected only in cold-acclimated leaves. Moreover, NP4, NP5, NP6, NP8, and NP9 all accumulated to higher levels in cold-acclimated leaves, as indicated by the intensity of the Coomassie brilliant blue stain when equal volumes of apoplastic extracts from both cold-acclimated and nonacclimated rye leaves were loaded on the native-polyacrylamide gel (Fig. 1). Only NP2 was found to accumulate to high levels in the apoplast of nonacclimated leaves.

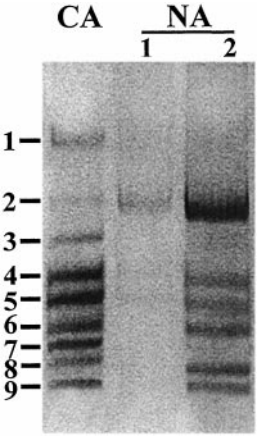

Figure 1. Separation of apoplastic proteins by native-PAGE. Apoplastic proteins were extracted from cold-acclimated (CA) and nonacclimated (NA) rye leaves by vacuum infiltration followed by centrifugation. The apoplastic proteins were separated from equal volumes of unconcentrated apoplastic extracts (lanes CA and NA, 1) and from equal amounts (10 μg) of apoplastic proteins (lanes CA and NA, 2) in an 8% continuous native-polyacrylamide gel. The gel was stained with Coomassie brilliant blue R-250. Numbers on the left refer to individual native apoplastic proteins.

Comparison of Apoplastic Polypeptides

When cold-acclimated and nonacclimated apoplastic proteins were denatured and separated by SDS-PAGE, 13 polypeptides were present in nonacclimated apoplastic extracts, with apparent molecular masses of 144, 97, 36, 35, 34, 33, 32, 28, 26, 25, 16 (doublet), and 14 kD (Fig. 2A, lane 3), whereas only 7 polypeptides were found in cold-acclimated apoplastic extracts, with molecular masses of 144, 35, 32, 28, 25, 16, and 14 kD (Fig. 2A, lane 1), when equal amounts of protein were loaded on the SDS-PAGE gel. Both cold-acclimated and nonacclimated apoplastic proteins contained 32- and 35-kD GLPs, a 35-kD CLP, and a 25-kD TLP, as detected positively on immunoblots probed with anti-GLP (Fig. 2B), anti-CLP (Fig. 2C), and anti-TLP antisera (Fig. 2D). No GLPs, CLPs, or TLPs were detected in nonacclimated apoplastic extracts when equal volumes of unconcentrated cold-acclimated and nonacclimated extracts were loaded onto the gels and immunoblots (Fig. 2, B–D, lanes 1 and 2).

Analysis of the Composition of Apoplastic NPs

The individual proteins shown in Figure 1 were eluted from the native gels, concentrated by ultrafiltration, and

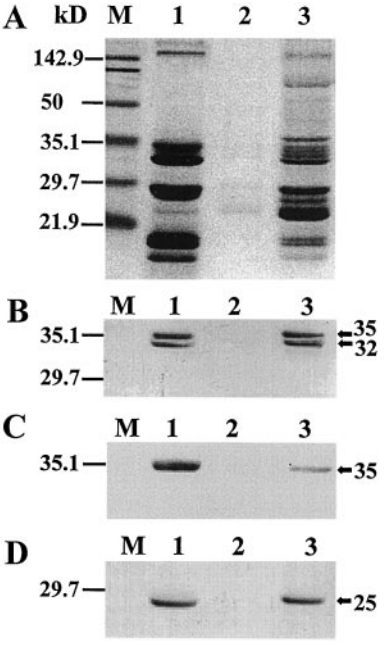

Figure 2. Examination of polypeptides in cold-acclimated and nonacclimated apoplastic extracts by SDS-PAGE and immunoblotting. A, SDS-PAGE (15% acrylamide) of equal volumes of crude cold-acclimated and nonacclimated apoplastic extracts (lanes 1 and 2, respectively) and equal amounts (5 μg) of cold-acclimated and nonacclimated apoplastic proteins (lanes 1 and 3, respectively). Polypeptides were stained with Coomassie brilliant blue R-250. Low-range prestained SDS-PAGE molecular-mass standards from Bio-Rad are shown on the left (lanes M). SDS-PAGE gels with the same sample-loading scheme shown in A were blotted and probed with anti-GLP antiserum (B), anti-CLP antiserum (C), and anti-TLP antiserum (D). Positive immunodetection of polypeptides is indicated by arrows on the right.

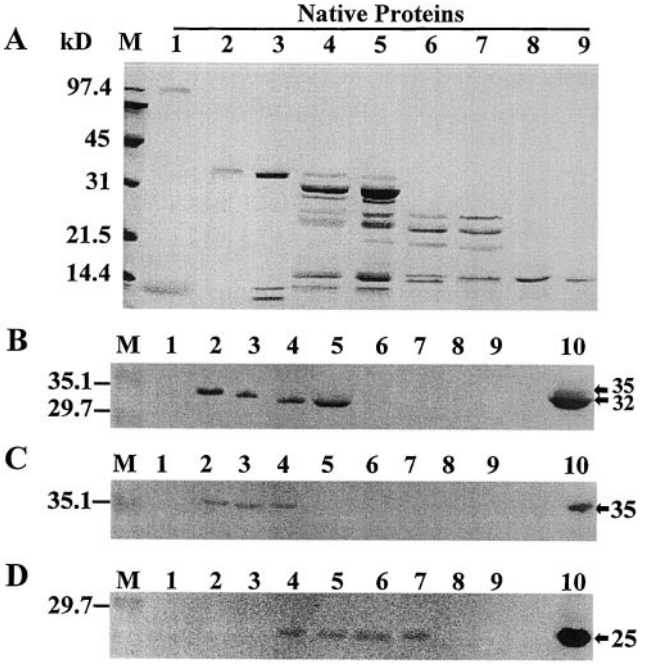

Figure 3. Examination of native apoplastic proteins by SDS-PAGE and immunoblotting. A, Individual cold-acclimated apoplastic proteins were eluted from the native gel shown in Figure 1, denatured, separated by 15% SDS-PAGE, and stained with Coomassie brilliant blue R-250. Equal amounts of protein (5 μg) were loaded on each lane. Lanes 1 to 9, NP1 to NP9. Low-range prestained SDS-PAGE molecular-mass standards from Bio-Rad are shown on the left (lanes M). SDS-PAGE gels loaded with equal amounts (1 μg per lane) of individual native apoplastic proteins and with crude cold-acclimated apoplastic extract in lanes 10 were blotted and probed with anti-GLP antiserum (B), anti-CLP antiserum (C), and anti-TLP antiserum (D). Positive immunodetection of polypeptides in apoplastic extracts is indicated by arrows on the right.

then denatured and examined by SDS-PAGE. All of the NPs except NP8 and NP9 contained multiple polypeptides when electrophoresed by denaturing SDS-PAGE (Fig. 3A). NP1 was composed of two polypeptides (12 and 97 kD). NP2 contained two polypeptides that migrated as a doublet with a molecular mass of 35 kD. NP3 showed four polypeptides, singlets of 10 and 12 kD and a doublet of 35 kD. NP4 showed seven polypeptides of 12, 14, 24, 25, 30, 32, and 35 kD. NP5 was composed of eight polypeptides with molecular masses of 12, 14, 22, 24, 25, 30, 32, and 35 kD. NP6 showed five polypeptides (13, 15, 22, 23, and 25 kD). NP7 showed four polypeptides (15, 22, 23, and 25 kD). NP8 and NP9 exhibited only one 15-kD polypeptide.

Analysis of NPs by Immunoblotting and Immunoprecipitation

The composition of each NP was further examined by immunoblotting. The apoplastic proteins initially separated by native-PAGE were denatured and separated by SDS-PAGE (Fig. 3A) and were then blotted and probed with antisera against GLPs (Fig. 3B), CLPs (Fig. 3C), and TLPs (Fig. 3D). The specificities of these antisera were

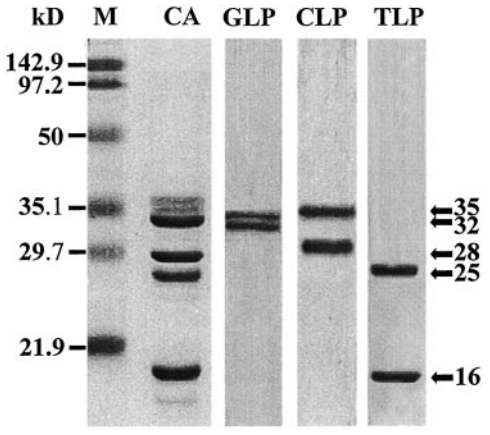

Figure 4. Specificities of anti-GLP, anti-CLP, and anti-TLP antisera. CA, Cold-acclimated apoplastic proteins (5 μg) were denatured, separated by 15% SDS-PAGE, and stained with Coomassie brilliant blue R-250. Similar gels were blotted and probed with antisera at a dilution of 1:3. Lane GLP was probed with anti-GLP antiserum, lane CLP was probed with anti-CLP antiserum, and lane TLP was probed with anti-TLP antiserum. Low-range prestained SDS-PAGE molecular-mass standards from Bio-Rad (lane M) were used to determine the molecular masses (kD). The molecular mass of each polypeptide immunodetected by an antiserum is indicated on the right.

described previously (Antikainen et al., 1996). Antiserum raised against the denatured 32-kD GLP, used in a dilution of 1:2000, recognizes two polypeptides with molecular masses of 32 and 35 kD, both of which were identified as GLPs by amino-terminal amino acid sequencing (Hon et al., 1995). Anti-CLP antiserum recognizes only one 35-kD polypeptide at a dilution of 1:2000, although the 35- and 28-kD polypeptides were both identified as CLPs by Hon et al. (1995). The anti-CLP antiserum was raised against the native 35-kD CLP, which has a chitin-binding domain that is lacking in the 28-kD CLP. Antiserum raised against the denatured 25-kD TLP detects only one polypeptide with a molecular mass of 25 kD at a dilution of 1:10,000. At this dilution, the 16-kD polypeptide also identified as a TLP by Hon et al. (1995) is not detected. Each antiserum is specific to one type of AFP and does not cross-react with other apoplastic proteins. Within each type of AFP, the antiserum is more reactive with the protein against which it was raised.

As shown in Figure 3, polypeptides associated with NP2 and some of the polypeptides associated with NP3 were positively detected by anti-GLP and anti-CLP antisera. Polypeptides associated with NP4 were detected by anti-GLP, anti-CLP, and anti-TLP antisera. Polypeptides associated with NP5 were detected by anti-GLP and anti-TLP antisera, whereas those associated with NP6 and NP7 were detected only by anti-TLP antiserum. In addition, NPs separated by nondenaturing gel electrophoresis were directly transferred to nitrocellulose membranes and probed with antisera against cold-induced winter rye GLP, CLP, and TLP. The results of these experiments confirmed the immunoblotting results obtained with SDS-PAGE (data not shown).

To examine further whether the observed cold-induced GLP, CLP, and TLP were in fact physically associated, two sets of experiments were conducted. Because these experiments required the use of higher antiserum concentrations, the specificity of each of the three antisera was determined using a dilution of 1:3 in immunoblots of denatured apoplastic polypeptides from cold-acclimated plants. Under these conditions, the anti-GLP antiserum detected two polypeptides with apparent molecular masses of 32 and 35 kD, the anti-CLP antiserum detected two polypeptides at 28 and 35 kD, and the anti-TLP antiserum detected two polypeptides at 16 and 25 kD (Fig. 4). In the first experiments, glucanase activity in cold-acclimated apoplastic extracts was assayed after incubating cold-acclimated apoplastic extracts with specific rye anti-GLP, anti-CLP, or anti-TLP antiserum. As shown in Figure 5, glucanase activity was dramatically inhibited in the presence of anti-GLP antiserum. Antisera produced against cold-induced CLP and TLP also inhibited glucanase activity by 93% and 95%, respectively. In other experiments, chitinase activity in the apoplastic extracts was inhibited by adding antiserum against GLP, CLP, or TLP (data not shown). In the second experiments, immunoprecipitation of AFPs by anti-GLP, anti-CLP, and anti-TLP antisera was examined (Fig. 6). The anti-GLP antiserum precipitated not only 32- and 35-kD GLPs but also a 35-kD CLP and a 25-kD TLP, as evident in the results from SDS-PAGE (Fig. 6A, lane 1) and immunoblotting (Fig. 6, B–D, lane 1). Different groups of polypeptides were immunoprecipitated by anti-CLP antiserum (Fig. 6, lanes 2) and anti-TLP antiserum (Fig. 6, lanes 3), but each of these antisera also precipitated a GLP, a CLP, and a TLP (Fig. 6, B–D).

Purification and Characterization of NP3

The separation of native and denatured proteins presented in Figures 1 and 3 could result from the comigration of proteins similar to GLPs, CLPs, and/or TLPs. To test this possibility, we isolated one complex from apoplastic ex-

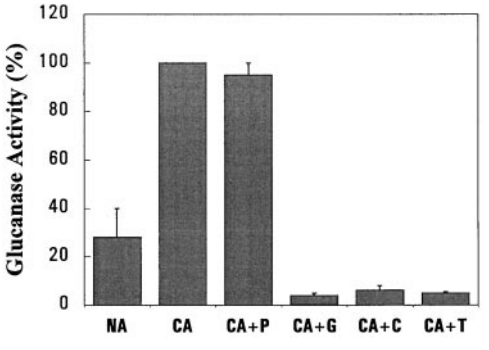

Figure 5. Inhibition of glucanase activity by antisera produced against AFPs. Glucanase activity was assayed in nonacclimated (NA) and cold-acclimated (CA) apoplastic extracts and in cold-acclimated extracts incubated with antisera produced against cold-induced GLP (G), CLP (C), and TLP (T), or with preimmune serum (P). The glucanase specific activity was normalized as a percentage of the specific activity present in the cold-acclimated apoplastic extract and is shown as the mean ± SE (n = 3).

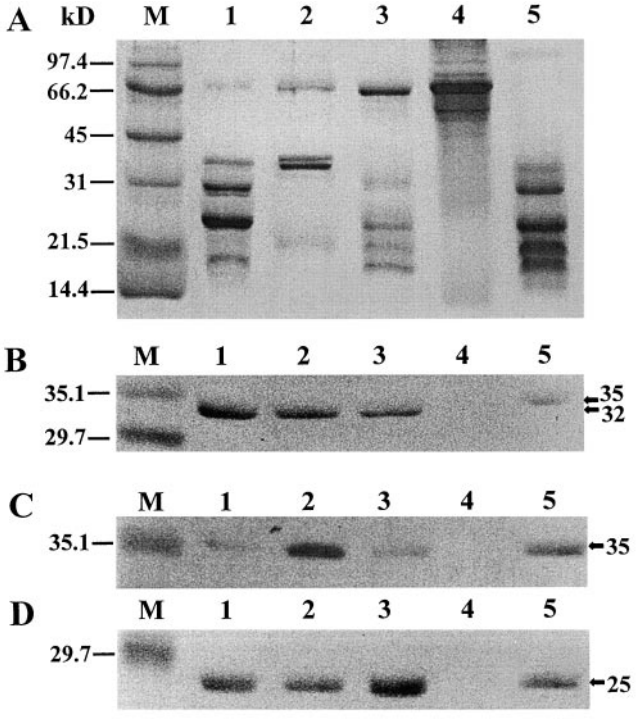

Figure 6. Immunoprecipitation of native AFPs by antisera produced against specific AFPs. Equal amounts (5 μg per lane) of proteins immunoprecipitated by anti-GLP (lanes 1), anti-CLP (lanes 2), or anti-TLP (lanes 3) antiserum, or by preimmune serum (lanes 4), were denatured and separated by 15% SDS-PAGE. Cold-acclimated apoplastic extract is shown as a positive control in lanes 5. A, Gel stained with Coomassie brilliant blue R-250. Gels were blotted and probed with anti-GLP antiserum (B), anti-CLP antiserum (C), and anti-TLP antiserum (D). Low-range prestained SDS-PAGE molecular-mass standards from Bio-Rad (lanes M) were used to determine the molecular masses (kD).

tracts of cold-acclimated rye leaves by chitin-affinity chromatography (Huynh et al., 1992; Hon et al., 1994). With this procedure, only chitinases or lectins with a chitin-binding domain specifically bind to the colloidal chitin used to pack the affinity column. By changing the pH of the washing buffer from 8.0 to 4.5, the nonspecifically bound proteins were washed off the column. The specifically bound chitinases were then washed off the column using 20 mM acetic acid, pH 3.0. In our experiments, only one protein was bound specifically to the chitin-affinity column. When separated by native-PAGE, this protein had an R_F value similar to that of NP3 in the crude cold-acclimated apoplastic extract (Fig. 7A). When denatured and separated by SDS-PAGE (Fig. 7B), this purified protein was composed of four polypeptides with molecular masses of 10, 12, 35, and 35 kD. The two 35-kD polypeptides were positively detected by antisera produced against cold-induced winter rye GLP and CLP (Fig. 7C).

Antifreeze Activities of NPs

The antifreeze activities of individual NPs eluted from native gels were compared with NP3 purified by chitin-affinity chromatography, crude apoplastic extract from nonacclimated leaves, and crude apoplastic extract from cold-acclimated leaves. The protein concentration of each sample was adjusted to about 0.5 mg mL^{-1}. As summarized in Table I, the highest antifreeze activity was found in the cold-acclimated crude apoplastic extract, which was composed of a mixture of all AFPs (rated 5). The next highest antifreeze activity was found in NP4, which contained GLP, CLP, and TLP (rated 4). This was followed by NP2 and NP3, which contained CLP and GLP (rate 3); NP5, which contained GLP and TLP (rated 3); and NP6 and NP7, which contained TLP (rated 2). NP1, NP8, and NP9 and crude apoplastic extract from nonacclimated leaves did not have any antifreeze activity (rated 0).

At this time, we cannot explain why nonacclimated apoplastic extracts lack antifreeze activity after they have been concentrated, because they contain NP2, NP4, NP5, and NP6 (Fig. 1), all of which exhibit antifreeze activity when isolated from cold-acclimated extracts (Table I). It may be that different isozymes of GLPs, CLPs, and/or TLPs accumulate at 20°C or that the proteins produced at 5°C are posttranslationally modified in some way to acquire antifreeze activity. Either of these possibilities could explain the differences in polypeptide composition between cold-acclimated and nonacclimated extracts observed on SDS-PAGE (Fig. 2).

DISCUSSION

Association of Winter Rye AFPs in Vivo

Our biochemical and immunological evidence shows that apoplastic extracts from cold-acclimated winter rye leaves contain nine proteins (Fig. 1). Seven of these are composed of multiple polypeptides when denatured and separated by SDS-PAGE (Fig. 3A), and, surprisingly, GLPs, CLPs, and TLPs are associated with six of them (Fig. 3, B–D). The association of glucanase with other proteins was observed previously (Ballance and Manners, 1978; Ji and Kuc, 1995). For example, native β-1,3-glucanase purified from germinated rye by ion-exchange chromatography on

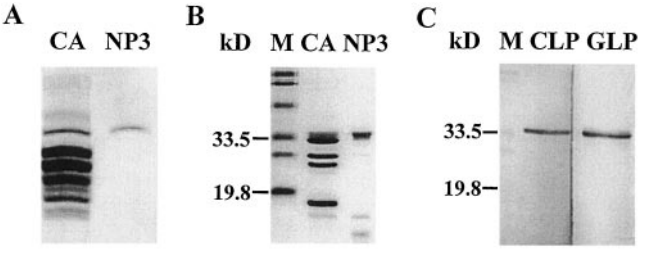

Figure 7. Purification and identification of NP3. Cold-acclimated (CA) rye apoplastic extract and purified NP3 were examined by native-PAGE (A), SDS-PAGE (B), and immunoblotting (C). NP3 was purified from rye apoplastic extract by chitin-affinity chromatography. Low-range prestained SDS-PAGE molecular-mass standards from Bio-Rad (lanes M) were used to determine the molecular masses (kD). The gels in A and B were stained with Coomassie brilliant blue R-250. Anti-GLP and anti-CLP antisera were used to detect GLP and CLP, respectively.

Table I. *Comparison of antifreeze activity of individual native proteins with the activity of crude apoplastic extracts from cold-acclimated and nonacclimated plants*

The individual proteins were separated by native-PAGE (Fig. 1), eluted, and assayed for antifreeze activity. Antifreeze activity of each sample was rated from 0 to 5 based on the shape of ice crystals grown in solution (see "Materials and Methods"), with 5 representing the highest activity and 0 representing no activity. The protein concentration of each sample was determined using the Bradford (1976) method, as modified by Bio-Rad, and is presented as the mean ± SE ($n = 3$).

Sample	Protein Concentration	Antifreeze Activity Rating
	mg mL^{-1}	
Apoplastic extract		
Cold-acclimated	0.45 ± 0.12	5
Nonacclimated	0.56 ± 0.07	0
NP1	0.40 ± 0.09	0
NP2	0.50 ± 0.14	3
NP3	0.45 ± 0.10	3
NP4	0.52 ± 0.08	4
NP5	0.42 ± 0.08	3
NP6	0.48 ± 0.09	2
NP7	0.50 ± 0.10	2
NP8	0.45 ± 0.08	0
NP9	0.52 ± 0.15	0

DEAE- and CM-cellulose followed by gel filtration on Bio-Gel P-60 was associated with three unidentified proteins (Ballance and Manners, 1978). Moreover, an acidic β-1,3-glucanase extracted from cucumber leaves infected with tobacco necrosis virus was associated with a class III chitinase because both enzymes migrated together on native-PAGE (Ji and Kuc, 1995).

It is possible that, by chance, the different proteins have the same mobility on a native gel, so they form a single band on the gel but migrate as several polypeptides on SDS-PAGE. To distinguish between these possibilities, NP3 (Fig. 7) was isolated by chitin-affinity chromatography and electrophoresed using the native-gel system in the absence of other apoplastic proteins. Only one NP was visible (Fig. 7A), and it contained both GLP and CLP, as determined by immunoanalysis (Fig. 7C). The binding between GLP and CLP appeared to be strong because GLP and CLP remained associated when other proteins were washed off the chitin-affinity column using solutions with pHs ranging from 8.0 to 4.8. The physical association among GLP, CLP, and TLP was also evident in the results of immunoinhibition experiments in which β-1,3-glucanase activity in cold-acclimated crude apoplastic extracts was dramatically inhibited by the presence of anti-GLP, anti-CLP, or anti-TLP antiserum, but not by preimmune serum (Fig. 5), and in immunoprecipitation experiments in which anti-GLP, anti-CLP, or anti-TLP antiserum precipitated all three AFPs (GLPs, CLPs, and TLPs) from cold-acclimated apoplastic extracts (Fig. 6). We interpret these results to indicate that all of these polypeptides are exposed on the surface of the NP complex. Alternatively, if the complexes are small, then the binding of any one of the antisera may hinder access of the polymeric substrate to the active site of the glucanase.

Why are there so many different NPs in the apoplastic extract? We hypothesize that the individual NPs are produced by different cell types. For example, immunolocalization studies of AFPs in winter rye revealed that all three classes of AFPs are localized in the epidermis of cold-acclimated leaves (Antikainen et al., 1996). Thus, NP4, NP5, NP6, and/or NP7 may be produced by epidermal cells. In contrast, just two classes of AFPs, GLPs and CLPs, accumulate in cell walls surrounding the intercellular spaces in the mesophyll (Antikainen et al., 1996; Pihakaski-Maunsbach et al., 1996). We would expect NP2 and NP3 to be secreted by mesophyll cells. Therefore, each NP identified in Figure 1 may have a different, tissue-specific location within the apoplast of a rye leaf.

Other investigators have also reported tissue-specific locations of glucanase and chitinase. In tomato plants, pathogen-induced β-1,3-glucanase and chitinase were both found in the abaxial epidermal layer near the stomata (Wubben et al., 1993). Ethylene-induced chitinase and β-1,3-glucanase have also been localized together in abaxial epidermal cells and in parenchymal cells adjacent to vascular strands in bean leaves (Mauch et al., 1992). Additional evidence for the physical association between β-1,3-glucanase and chitinase was obtained by immunolocalization of β-1,3-glucanase and chitinase in the large, electron-dense aggregates located in the vacuoles of lower epidermal cells of ethylene-treated bean leaves (Mauch et al., 1992). The common compartmental location of glucanase and chitinase is consistent with our hypothesis that the two enzymes are physically associated with each other.

The arrangement of specific proteins and enzymes as part of functional complexes has been found in many systems. For example, the cellulose (mainly β-1,4-glucanase) activity of many cellulolytic bacteria occurs in discrete, multifunctional, multienzyme complexes called cellulosomes. These organized complexes account for the efficient solubilization of insoluble cellulose (Bayer et al., 1994). A second example of a multienzyme complex is acetyl-CoA carboxylase, which consists of biotin carboxylase and a biotin-carboxyl carrier protein and is a regulatory enzyme of fatty acid synthesis (Roesler et al., 1996).

Although β-1,3-glucanase and chitinase are encoded by two different small gene families (Linthorst, 1991), the expression of the two genes is often coordinately regulated upon pathogen infection and exposure to other environmental stresses such as wounding, drying, and flooding (Ohashi and Ohshima, 1992; Stintzi et al., 1993). Glucanase and chitinase enzymatic activities increase concomitantly in many plants not only in response to pathogen attack but also in response to pathogen-derived elicitors and the plant hormone ethylene (Mauch et al., 1992). Moreover, it has been reported that β-1,3-glucanase or chitinase purified from pea cannot inhibit the growth in culture of most of the fungi tested when used individually. However, a combination of these enzymes effectively inhibits the growth of most fungi tested (Mauch et al., 1988), which suggests that the two enzymes act synergistically in plant defense.

It is possible that GLPs, CLPs, and TLPs play a synergistic role in improving the winter survival of winter rye plants. First, β-1,3-glucanase and chitinase activities are

both high in apoplastic extracts from cold-acclimated winter rye leaves; therefore, they may act together to provide resistance to pathogens (Hon et al., 1995). Second, GLPs, CLPs, and TLPs may work in concert to modify the growth of intercellular ice as part of the mechanism of freezing tolerance. These proteins are all present in the apoplast of rye leaves after cold acclimation (Hon et al., 1995), and they are located in the epidermis and in the cell walls lining the intercellular spaces of cold-acclimated leaves, where they may interact with ice (Antikainen et al., 1996; Pihakaski-Maunsbach et al., 1996). The GLPs and CLPs appear to be physically associated with each other (Fig. 7), which is significant because the antifreeze activity of complexes that contain both GLPs and CLPs is higher than that of complexes that lack these proteins (Table I).

Possible Roles of AFP Complexes

The general mechanism of action of AFPs can be explained by the adsorption-inhibition theory described in detail by Raymond and DeVries (1977). An ice crystal normally grows as a broad front with a low radius of curvature. However, when ice crystals are grown in a solution containing AFPs, the AFPs interact with ice in two unique ways. First, AFPs adsorb onto the nonbasal planes of ice at the ice-water interface (Raymond et al., 1989) and exert a concentration-dependent effect on ice-crystal growth features (DeVries, 1986). Second, AFPs adsorbing onto the ice surface block the binding of additional water molecules, which creates an ice-crystal surface with many highly curved fronts and a high surface free energy. Consequently, the growth of these fronts is halted because it is less energetically favorable for water molecules to bind to this surface. The temperature must be lowered further to decrease free energy before crystal growth proceeds. As a result, the freezing point of the solution is depressed.

Theoretically, one way to increase the effectiveness of an AFP is to increase the size of the protein so that it blocks a greater area on the ice-crystal surface. Wu et al. (1991a) demonstrated that this size effect does occur. They showed that an insect AFP conjugated with rabbit anti-AFP IgG, which has no antifreeze activity by itself, plus goat anti-rabbit IgG, which also has no antifreeze activity by itself, exhibits greater antifreeze activity than the insect AFP alone. Furthermore, they showed that a second protein isolated from insect hemolymph can bind to the insect AFP and enhance its activity (Wu et al., 1991b). Thus, the high level of antifreeze activity observed in crude hemolymph extracts obtained from overwintering larvae probably requires the interaction of the insect AFP (12–22 kD) and its activator protein (70 kD).

The AFPs in winter rye may function in a fashion similar to the insect AFP plus its activator protein. By forming complexes composed of GLPs, CLPs, and/or TLPs, the AFPs may block a larger area of the ice surface, thus making it more difficult for the ice to overgrow the complex(es). As a result, the growth of ice crystals is inhibited to a greater extent and antifreeze activity increases. The fact that the apoplastic extract, which is a mixture of AFPs, has the highest antifreeze activity, followed by the complex containing GLP, CLP, and TLP, and then complexes consisting of GLP and TLP or GLP and CLP (Table I), provides indirect evidence to support our hypothesis.

Physical damage caused by ice can occur in frozen tissues when small ice crystals condense into larger ones, a process known as recrystallization (Knight and Duman, 1986). Although recrystallization occurs slowly during prolonged freezing at very low temperatures, it can happen very quickly at temperatures near the melting point of ice. In nature, the inhibition of ice recrystallization may be the primary role of AFPs in freezing-tolerant organisms (Knight and Duman, 1986). Winter rye leaves form extracellular ice during winter (Pearce, 1988) and are exposed to fluctuating subzero temperatures that promote the recrystallization of ice. Winter rye AFPs inhibit ice recrystallization effectively at very low concentrations (25 μg protein L^{-1}) (Griffith and Antikainen, 1996). The ability of rye AFPs to inhibit ice recrystallization at low protein concentrations may be related to both the size of proteins and the presence of multiple ice-binding sites on each protein. For example, it is possible that each AFP complex can interact with more than one ice crystal, because each of the components of the complex is able to interact with ice. In summary, we conclude that winter rye AFPs form oligomeric complexes in the apoplast that enhance the ability of these proteins to inhibit the growth and recrystallization of ice.

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