Plant Physiology, October 1999, Vol. 121, pp. 665–673, www.plantphysiol.org © 1999 American Society of Plant Physiologists

Snow-Mold-Induced Apoplastic Proteins in Winter Rye Leaves Lack Antifreeze Activity[1]

Mervi Hiilovaara-Teijo[2]*, Asko Hannukkala, Marilyn Griffith, Xiao-Ming Yu, and Kaarina Pihakaski-Maunsbach[3]

Department of Biology, Laboratory of Plant Physiology and Molecular Biology, FIN–20014 University of Turku, Turku, Finland (M.H.-T., K.P.-M.); Agricultural Research Centre, Institute of Plant Protection, FIN–31600 Jokioinen, Finland (A.H.); and Department of Biology, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1 (M.G., X.-M.Y.)

During cold acclimation, winter rye (*Secale cereale* L.) plants secrete antifreeze proteins that are similar to pathogenesis-related (PR) proteins. In this experiment, the secretion of PR proteins was induced at warm temperatures by infection with pink snow mold (*Microdochium nivale*), a pathogen of overwintering cereals. A comparison of cold-induced and pathogen-induced proteins showed that PR proteins accumulated in the leaf apoplast to a greater level in response to cold. The PR proteins induced by cold and by snow mold were similar when separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and examined by immunoblotting. Both groups of PR proteins contained glucanase-like, chitinase-like, and thaumatin-like proteins, and both groups exhibited similar levels of glucanase and chitinase activities. However, only the PR proteins induced by cold exhibited antifreeze activity. Our findings suggest that the cold-induced PR proteins may be isoforms that function as antifreeze proteins to modify the growth of ice during freezing while also providing resistance to the growth of low-temperature pathogens in advance of infection. Both functions of the cold-induced PR proteins may improve the survival of overwintering cereals.

In the northern boreal ecosystem, overwintering cereals must survive subzero temperatures and long-lasting snow cover, which exposes them to injury caused both by freezing and by snow molds (Gaudet and Laroche, 1997). Snow molds are a diverse group of low-temperature parasitic fungi that infect winter cereals such as wheat, barley, and rye, as well as numerous grasses, when protected by snow cover (Jamalainen, 1974). Common snow molds on graminaceous plants include pink snow mold (*Microdochium nivale*), speckled snow mold (*Typhula* spp.), and Sclerotinia snow mold (*Sclerotinia borealis*).

Although plants growing under stressful conditions often become more susceptible to fungal diseases, some plants exhibit a phenomenon called cross-adaptation, whereby exposure to one stress provides tolerance to other stresses (Sabehat et al., 1998; Hiilovaara-Teijo and Palva, 1999). For example, Tronsmo (1984, 1985, 1993) showed that overwintering perennial grasses become more resistant to fungal diseases such as snow molds, powdery mildews, leaf spots, and rusts once the plants have undergone acclimation to low temperatures. In timothy grass (*Phleum pratense*) and cocksfoot (*Dactylis glomerata*), the increase in freezing tolerance that occurs during cold acclimation is correlated with the acquisition of greater resistance to snow molds (Tronsmo, 1984, 1993). Moreover, the genotypic correlation between freezing tolerance and snow mold resistance is approximately 1 in half-sibling families of cocksfoot, which suggests that the same genetic trait(s) may be involved (Tronsmo, 1993). Nakajima and Abe (1996) also showed that winter wheat must be grown at low temperatures to become snow mold resistant. At this time, the precise relationship between freezing tolerance and snow mold resistance is not well understood (Gaudet, 1994).

One trait that may be shared in the acquisition of both disease resistance and freezing tolerance is the production of pathogenesis-related (PR) proteins that are involved in induced disease resistance. Among the PR proteins with antifungal activities that are induced in plants by pathogens are glucanases, chitinases, thaumatin-like proteins (TLPs), and ribosome-inactivating proteins (Stintzi et al., 1993; Collinge et al., 1994). Moreover, Tronsmo et al. (1993) observed that PR proteins such as chitinases accumulate in barley plants not only after inoculation with powdery mildew, but also in response to cold temperatures. This phenomenon has been reported in a number of plants. When exposed to low temperatures, *Solanum commersonii* expresses a gene encoding the TLP osmotin (Zhu et al., 1993). Winter rye (*Secale cereale* L.), wheat, and barley leaves accumulate proteins similar to PR proteins (Hon et al., 1995; Antikainen and Griffith, 1997). Bermudagrass expresses a gene encoding chitinase (Gatschet et al., 1996) and carrot expresses a gene encoding a polygalacturonase inhibitor protein (Worrall et al., 1998; Meyer et al., 1999). Tamás et al. (1997) also detected the accumulation of three unidentified proteins in the apoplast of barley leaves in response to powdery mildew attack as well as during low-temperature stress. One interpretation of these results

[1] This work was supported by research grants from the Academy of Finland (project no. 29502 to K.P.-M.) and from the Natural Science and Engineering Research Council of Canada (to M.G.).

[2] Present address: Dr. Mervi Hiilovaara-Teijo (former name Antikainen), Department of Biosciences, Division of Genetics, P.O. Box 56, FIN–00014 University of Helsinki, Finland.

[3] Present address: Department of Cell Biology, Institute of Anatomy, University of Aarhus, DK–8000 Aarhus C, Denmark.

* Corresponding author; e-mail Mervi.Hiilovaara-Teijo@Helsinki.Fi; fax 358–9–708–59079.

is that the metabolic response of overwintering plants to low-temperature pathogens may be too slow at subzero temperatures to provide an adequate defense. Thus, the accumulation of PR proteins during cold acclimation may be a protective measure produced by overwintering plants in advance of pathogen invasion.

In winter rye, six PR proteins accumulate to high levels in the leaf apoplast when plants are exposed to low temperature (Marentes et al., 1993; Hon et al., 1995). Two of these proteins are similar to endo-β-1,3-glucanases (GLPs), two are similar to endochitinases (CLPs), and two are TLPs (Hon et al., 1995). However, these PR proteins are unusual because they also exhibit antifreeze activity, the ability to modify the growth of ice (Griffith et al., 1992; Hon et al., 1994; Antikainen and Griffith, 1997). Because the antifreeze proteins (AFPs) bind to the surface of ice crystals, they are thought to inhibit the recrystallization of extracellular ice in frozen plants exposed to fluctuating and/or prolonged subzero temperatures (Griffith and Antikainen, 1996). As a result, the accumulation of AFPs is correlated with increased freezing tolerance in winter rye leaves (Marentes et al., 1993) and with higher rates of winter survival in winter wheat plants (Chun et al., 1998).

We do not know whether the winter rye AFPs that are similar to PR proteins retain their antifungal activities, or if they have been modified to function solely as AFPs. Therefore, we decided to study the accumulation of AFPs from the leaves of rye plants infected with a pathogen at warm temperatures and to compare those PR proteins with the AFPs that accumulate in response to low temperature. Winter rye leaves were inoculated with pink snow mold. The objectives of this study were: (a) to examine whether apoplastic protein secretion is enhanced as a response to snow mold infection in winter rye leaves, (b) to determine whether similar apoplastic proteins accumulate in response to cold temperature and snow mold infection, and (c) to determine if the secreted proteins possess antifreeze activity and/or glucanase and chitinase activities in both cases. Our results show that while the two groups of proteins are similar in number and composition, they are different in activity. PR proteins induced by snow mold at warm temperatures exhibit enzymatic activities but lack antifreeze activity, whereas the AFPs similar to PR proteins induced by cold temperatures exhibit both enzymatic and antifreeze activities.

MATERIALS AND METHODS

Snow Mold Inoculum and Inoculations

Pink snow mold (*Microdochium nivale*) isolates were collected from three different locations in Finland (Kokemäki, Mietoinen, and Pälkäne) and grown on potato dextrose agar (Biokar Diagnostics, Beauvais, France) for 10 to 14 d at 18°C under continuous light. Spores were then collected in 3 mL of distilled water with a glass rod. The spore concentration was adjusted to 100,000 spores mL^{-1} distilled water. The spore suspension was frozen and stored at −20°C until inoculation. The inoculation was performed by spraying 1 mL of spore suspension per pot by using a high-pressure sprayer. After inoculation the plants were covered with moistened tissue paper and each pot was placed in plastic bag to maintain 100% moisture during the incubation (Fig. 1A). Noninfected control plants were grown under moistened tissue paper in plastic bags without snow mold inoculum.

Plant Materials and Growth Conditions

For the temperature treatments, winter rye (*Secale cereale* L. cv Musketeer) was planted in coarse vermiculite in 15-cm pots and grown in a controlled-environment growth chamber maintained at 20°C/16°C (day/night) with a 16-h photoperiod and a PPFD of 180 μmol of photons m^{-2} s^{-1} for 3 weeks; these plants are referred to as nonacclimated at 20°C (NA20, Table I). For cold acclimation, some of the pots of 1-week-old NA20 plants were transferred to a cold growth chamber maintained at 5°C/2°C with an 8-h photoperiod and a PPFD of 180 μmol of photons m^{-2} s^{-1} for 7 weeks (CA5, Table I) to reach the same physiological age as 3-week-old nonacclimated ones (Krol et al., 1984; Griffith and McIntyre, 1993).

For snow mold treatments, winter rye was grown in pots containing a fertilized peat-soil mixture in nonacclimating greenhouse conditions (18°C and a minimum 14-h photoperiod) until the two-leaf stage, which usually occurred in 2 to 3 weeks. Natural sunlight dictated the light regime in the greenhouse during the summer (May–August), whereas plants were grown during the winter with additional artificial light from 6 to 8 AM and again from 6 to 8 PM to maintain a minimum 14-h daylength. Half of the pots served as greenhouse controls and were grown without snow mold inoculum (NA18). The other half were inoculated at 18°C with the pink snow mold spore suspension (NA18 INF) and were incubated for at least 2 weeks until visible symptoms were apparent (Fig. 1B). Winter rye was also cold-acclimated under controlled conditions at 5°C/2°C for 5 weeks prior to snow mold inoculation to ensure the accumulation of all three classes of AFPs (Hon et al., 1995; Antikainen and Griffith, 1997). After cold conditioning, plants were transferred to 2°C with a 14-h photoperiod and 85 μmol of photons m^{-2} s^{-1} for an additional 4 to 5 weeks. Half of these plants were grown without snow mold inoculum (CA2) and half were incubated with snow mold until symptoms of the disease were clearly visible (CA2 INF). Moreover, nonacclimated plants without cold conditioning (growth conditions as in NA18) were transferred at the two-leaf stage to a growth chamber set at 2°C with a 14-h day and a PPFD of 85 μmol of photons m^{-2} s^{-1}. Half of these plants were grown without exposure to snow mold spores (NA2) and the other half were inoculated and incubated with snow mold for at least 4 weeks (NA2 INF).

Plants grown in vermiculite were watered with nutrient solution as needed (Hoagland and Arnon, 1950). The development of symptoms after snow mold inoculation was monitored visually, and the plants were harvested for apoplastic protein extractions when damage was clearly visible on the leaves. Noninfected control plants were harvested at the same time as infected ones.

Figure 1. Winter rye plants inoculated with pink snow mold showing damage on leaves. A, After inoculation, rye seedlings were incubated in plastic bags. The moisture was maintained near 100% RH with wet tissue paper. B, Snow mold caused yellowing lesions (arrowheads) on rye seedlings. In wet conditions lesions spread rapidly and the leaf tissue collapsed down. The damaged leaves had a pinkish color due to pigments produced by the pathogen.

Extraction of Apoplastic Proteins

Apoplastic proteins were extracted from all treatments according to the method of Hon et al. (1994). Leaf segments were vacuum-infiltrated with a solution containing 20 mM ascorbic acid and 20 mM $CaCl_2$ (pH 3.0), followed by centrifugation to recover the infiltrate. Protein concentrations in apoplastic extracts were determined using the Bradford protein assay with BSA as the standard (Bradford, 1976).

Assay of Antifreeze Activity

The antifreeze activity of apoplastic protein extracts was assayed using a nanoliter osmometer according to the method of Hon et al. (1994, 1995) to observe the morphology of ice crystals grown in solution. In water, ice crystals are circular discs; therefore, circular ice crystals indicate no antifreeze activity in the solution. In contrast, ice crystals grown in solutions containing AFPs are thicker, with distinctive hexagonal shapes, because AFPs adsorb onto the

Table I. *Summary of the experimental conditions (day/night temperatures and length of treatment) for growing winter rye seedlings and applying cold and snow mold treatments*

Plants were grown for the indicated times under non-acclimating and cold-acclimating conditions, and half of the plants were inoculated and incubated with snow mold. NA20, NA18, NA2, and CA2 represent control plants for CA5, NA18 INF, NA2 INF, and CA2 INF plants, respectively. The leaves were harvested for apoplastic extractions from at least three ($n = 3$) independent experiments.

Sample[a]	Growth	Cold Treatment	Snow Mold Treatment	Age at Harvest
NA20	20°C/16°C, 3 weeks	–	–	3 weeks
CA5	20°C/16°C, 1 week	5°C/2°C, 7 weeks	–	8 weeks
NA18	18°C/18°C, 5 weeks	–	–	5 weeks
NA18 INF	18°C/18°C, 2–3 weeks	–	18°C/18°C, 2–3 weeks	5 weeks
NA2	18°C/18°C, 2–3 weeks	2°C/2°C, 4–5 weeks	–	7–8 weeks
NA2 INF	18°C/18°C, 2–3 weeks	–	2°C/2°C, 4–5 weeks	7–8 weeks
CA2	20°C/16°C, 1 week	5°C/2°C, 5 weeks + 2°C/2°C, 4–5 weeks	–	10–11 weeks
CA2 INF	20°C/16°C, 1 week	5°C/2°C, 5 weeks	2°C/2°C, 4–5 weeks	10–11 weeks

[a] CA, cold-acclimated; INF, infected with snow mold; NA, non-acclimated.

prism faces of an ice crystal, thus inhibiting its growth along the *a*-axes but allowing it to grow along the *c*-axis. The formation of ice crystals shaped as hexagonal plates, columns, and bipyramids represents low, moderate, and high antifreeze activity, respectively (DeVries, 1986).

Protein Electrophoresis and Immunoblotting

Equal amounts of extracted apoplastic proteins were separated by SDS-PAGE (Laemmli, 1970) using 15% (v/v) polyacrylamide gels (7 × 7 × 0.15 cm). The polypeptides were stained with silver stain (Sambrook et al., 1989). Proteins were transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) and probed separately with antisera produced against three winter rye AFPs: the 32-kD GLP, the 35-kD CLP, and the 25-kD TLP (Antikainen et al., 1996). These rabbit polyclonal antibodies were used in dilutions of 1:10,000, 1:100, and 1:10,000, respectively. The immunoreaction was detected by alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad) with 5-bromo-4-chloro-3-indolylphosphate-toluidine salt (Bio-Rad), and nitroblue tetrazolium (Bio-Rad) as substrates.

Glucanase and Chitinase Assays

Endo-β-1,3-glucanase activity was determined by incubating various amounts of apoplastic protein extracts with 0.05 mL of laminarin (5%, w/v) at 37°C for 10 min; then, 0.3 mL of dinitrosalicylate reagent was added to the solution and it was boiled for 5 min. Endoglucanase activity was quantified as the release of reducing equivalents of 3,5-dinitrosalicylate by measuring A_{492} (Bernfeld, 1955). One katal was defined as the enzyme activity producing 1 mol of Glc equivalents s^{-1}.

The colorimetric assay for endochitinase was carried out using a modification of Boller et al. (1983) and Legrand et al. (1987). The reaction mixture contained 0.5 mg of colloidal chitin and various volumes of crude apoplastic extract in a final volume of 0.5 mL of 0.1 M sodium acetate (pH 5.2). This mixture was incubated on a test tube rotator at 37°C for 1 h. After incubation the tubes were centrifuged at 10,000*g* for 10 min. To 0.3 mL of the supernatant was added 0.02 mL of 3% (w/v) snail gut enzyme, the mixture was incubated at 37°C for 1 h, and then 0.1 mL of 0.6 M potassium tetraborate was added to the tubes before heating for 3 min. After rapid cooling, 1 mL of the reagent stock solution (10% [w/v] 4-[methylamino]benzaldehyde in glacial acetic acid and 11.5 M HCl; 87.5:12.5 [v/v]) diluted 1:2 with glacial acetic acid was added. After incubation at 37°C for 20 min, the amount of liberated *N*-acetyl-glucosamine (GlcNAc) was determined spectrophotometrically at 585 nm. One katal was defined as the enzyme activity producing 1 mol of GlcNAc equivalents s^{-1}. Endochitinase and endo-β-1,3-glucanase activities were expressed in nanokatals (nkat) or microkatals (μkat), respectively, per milligram of apoplastic protein or per gram fresh weight of leaf tissue.

Statistical Analysis

The Student's *t* test (SAS Institute, 1985) was used at the 5% level of significance to detect differences in apoplastic protein accumulation and enzyme activities between NA and CA plants, and between noninfected and snow-mold-infected plants. The results were obtained from at least three independent experiments.

RESULTS

Disease Symptoms

NA winter rye inoculated with snow mold at 18°C (NA18 INF) showed very severe disease symptoms and most of the leaves were wholly necrotic, flaccid, or rotted after 3 weeks of incubation (Fig. 1B). In contrast, leaves that were cold-acclimated prior to snow mold infection at 2°C (CA2 INF) showed fewer symptoms, even though mycelial growth was evident on the leaves, and brown, spherical sclerotia were visible. Because snow mold symptoms develop more slowly at low temperatures (Hömmö, 1994b), the same level of damage detected in NA rye after 2 to 3 weeks was achieved at 2°C only after 4 to 5 weeks. Therefore, NA and CA snow-mold-infected winter rye plants were of different ages when harvested for apoplastic protein extractions (Table I).

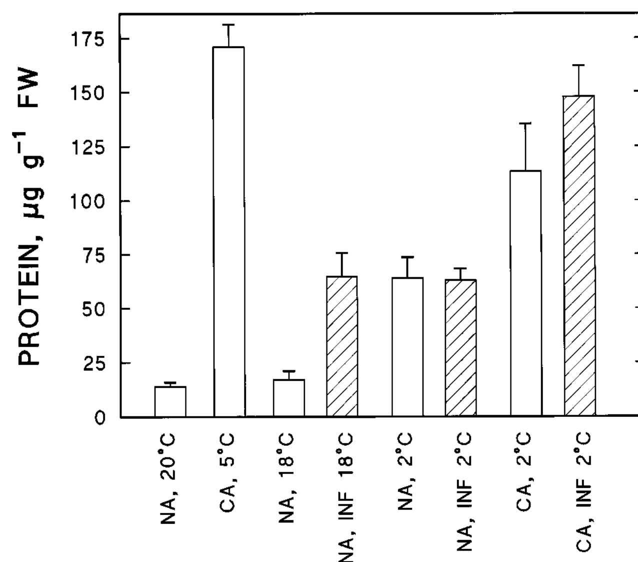

Figure 2. Apoplastic protein accumulation in winter rye leaves as a response to cold acclimation and to snow mold infection. As summarized in Table I, plants were grown under nonacclimating and cold-acclimating conditions, and half of the plants were inoculated and incubated with pink snow mold. Protein concentrations were measured in leaf apoplastic extracts obtained from at least three independent experiments. Data are presented as the means ± SE. The protein contents were significantly higher in cold-acclimated CA5 ($P < 0.001$, $n = 4$) and snow-mold-infected NA18 INF ($P < 0.01$, $n = 5$) plants than in NA20 and NA18 plants, respectively. The protein contents in snow-mold-infected NA2 INF and CA2 INF plants were not statistically different from those in the noninfected NA2 and CA2 plants, respectively. White bars, Noninfected plants; hatched bars, snow-mold-infected plants. FW, Fresh weight.

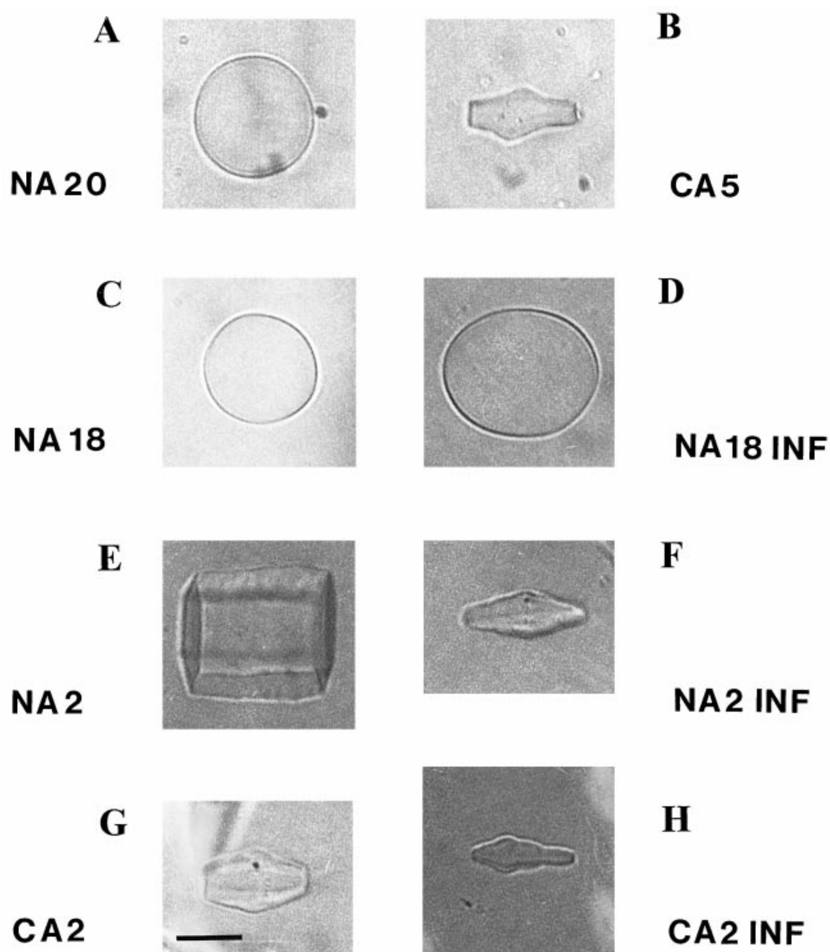

Figure 3. Antifreeze activity as shown by representative ice crystals grown in apoplastic extracts from all treatments described in Table I: A, NA20; B, CA5; C, NA18; D, NA18 INF; E, NA2; F, NA2 INF; G, CA2; and H, CA2 INF. Protein concentrations in all samples were adjusted to 1 mg mL^{-1} to ensure that the lack of antifreeze activity was not due to the low protein concentration. Ice crystal morphologies observed in protein solutions indicate differences in antifreeze activity: circular or rounded hexagonal discs (A, C, and D) indicate no antifreeze activity, whereas hexagons, hexagonal columns (E), and bipyramids (B, F, G, and H) represent low, moderate, and high antifreeze activity, respectively. In ice crystals A, C, and D, the *c*-axis is perpendicular to the plane of the photograph. In ice crystals B and E to H, the *c*-axis is parallel to the plane of the photograph. Magnification bar = 20 μm.

Apoplastic Protein Accumulation

We compared the accumulation of apoplastic proteins produced in snow-mold-infected and control plants with proteins produced by NA20 and CA5 plants that had already been well-characterized (Hon et al., 1994, 1995). Plants that were cold-acclimated for 7 weeks at 5°C (CA5) exhibited a statistically significant increase (12.2-fold) in the accumulation of apoplastic proteins compared with NA20 plants (Fig. 2). Correspondingly, infection with snow mold at warm temperature (NA18 INF) significantly increased the apoplastic protein content by 3.8-fold compared with healthy, noninfected plants (NA18). In contrast, infecting NA plants at a cold temperature (NA2 INF) did not increase the apoplastic protein content compared with noninfected winter rye grown under the same conditions (NA2, Fig. 2). Although low temperature and snow mold infection under nonacclimating conditions both induced an equivalent accumulation of apoplastic proteins, the effect was not additive. When winter rye was cold-acclimated before snow mold infection at 2°C (CA2 INF), the apoplastic protein concentration increased to a level comparable to that of CA5 plants (Fig. 2). Again, compared with the noninfected control plants (CA2), snow mold infection at low temperature (CA2 INF) did not enhance apoplastic protein accumulation significantly.

Antifreeze Activity

Apoplastic protein samples from each treatment were assayed for antifreeze activity. We found that the apoplastic proteins from NA plants (Fig. 3, A and C) and NA18 INF plants (Fig. 3D) did not modify the growth of ice crystals in vitro, because the ice crystals formed circular discs. Apoplastic proteins with the ability to modify ice formation accumulated only in winter rye leaves exposed to cold temperature (Fig. 3, B and E–H). Apoplastic extracts obtained from nonacclimated plants that were transferred to 2°C for 4 to 5 weeks formed ice crystals shaped like hexagonal columns (Fig. 3E), which indicated the presence of moderate antifreeze activity in the leaves. The highest antifreeze activity, shown by ice crystals shaped like hexagonal bipyramids that spiked to form needle-like crystals at lower temperatures (DeVries, 1986; Griffith et al., 1992), was measured in apoplastic extracts from plants that were cold-acclimated (Fig. 3B) or exposed to cold temperatures either at the same time (Fig. 3F) or before they were infected with snow mold at 2°C (Fig. 3H).

Characterization of Apoplastic Polypeptides

To characterize the qualitative changes in apoplastic extracts, the proteins were denatured, separated by SDS-

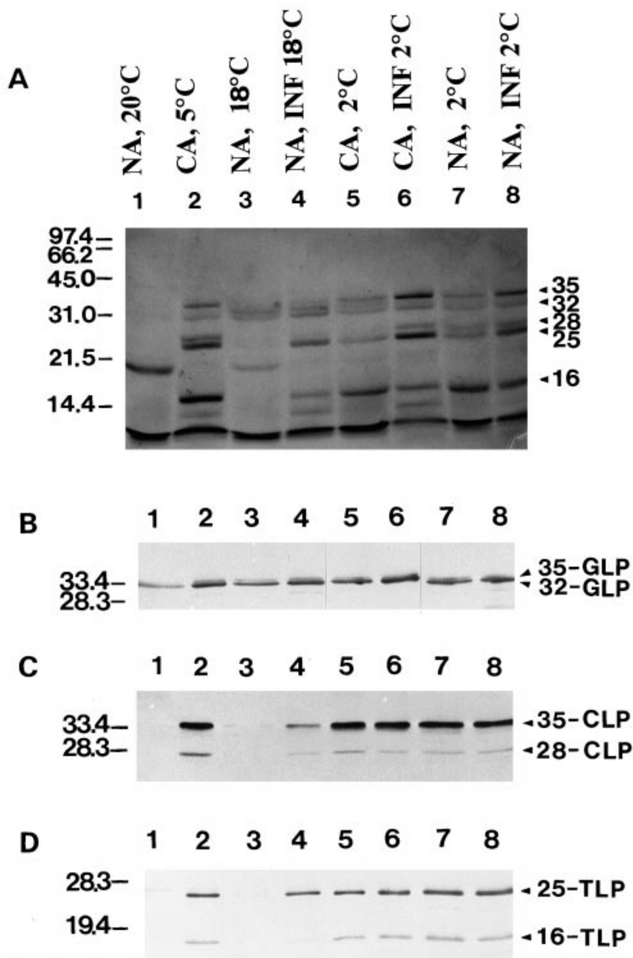

Figure 4. SDS-PAGE and immunodetection of PR proteins in NA, CA, noninfected, and snow-mold-infected (INF) winter rye. A, Equal amounts of polypeptides (2 μg of protein/lane) were separated from apoplastic extracts obtained from NA20 (lane 1), CA5 (lane 2), NA18 (lane 3), NA18 INF (lane 4), CA2 (lane 5), CA2 INF (lane 6), NA2 (lane 7), and NA2 INF (lane 8). The 15% (v/v) SDS-polyacrylamide gel was silver-stained. For immunoblotting, SDS-polyacrylamide gels loaded with equal amounts (1 μg per lane) of apoplastic protein were blotted and probed with anti-GLP antiserum (dilution 1:10,000) produced against winter rye 32-kD GLP (B), anti-CLP antiserum (dilution 1:100) produced against winter rye 35-kD CLP (C), and anti-TLP antiserum (dilution 1:10,000) produced against winter rye 25-kD TLP (D). Positive immunodetection of each of the corresponding polypeptides is indicated on the right. Numbers on the left refer to the low-molecular-mass markers in A, and prestained molecular-mass markers in B to D.

PAGE, and examined with antisera to AFPs from CA rye (Antikainen et al., 1996). These results show that polypeptides with the same molecular masses were produced in response to both cold temperature and snow mold infection. Furthermore, immunologically similar PR proteins (β-1,3-glucanases, chitinases, and TLPs; Hon et al., 1995) accumulated in response to both low temperature and pathogen stress (Fig. 4). Apoplastic extracts from NA20 plants exhibited only one GLP 32 kD in molecular mass (Fig. 4B, lane 1), whereas extracts from NA18 plants and all plants exposed to either low temperature and/or snow mold accumulated two GLPs of 35 and 32 kD (Fig. 4B, lanes 2–8). Anti-CLP antiserum recognized two CLPs, 28 and 35 kD, in apoplastic extracts of all rye plants exposed to low temperature or infected with snow mold, but no CLPs were observed in NA plants (Fig. 4C). Similarly, antiserum against the 25-kD rye TLP detected a 25-kD TLP in all rye plants treated with cold temperature or infected with snow mold (Fig. 4D, lanes 2 and 4–8), but no 25-kD TLP was observed in extracts from NA plants (Fig. 4D, lanes 1 and 3). The 16-kD TLP exhibited a different pattern in that it appeared only in plants that were exposed to cold temperatures (Fig. 4D, lanes 2 and 5–8).

Although immunoblotting results are not quantitative, an examination of the polypeptides separated by SDS-PAGE showed that the treatments that exhibited the greatest modification of ice crystal growth, CA5, NA2 INF, and CA2 INF (Fig. 3, B, F, and H), also showed the greatest increase in accumulation of the 35-, 25-, and 16-kD polypeptides (Fig. 4A, lanes 2, 8, and 6, respectively). In

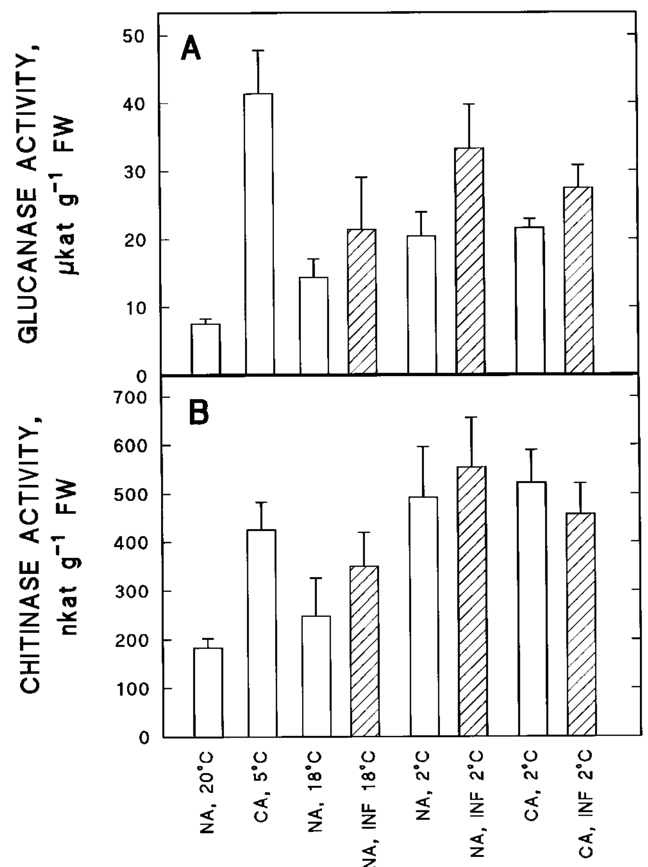

Figure 5. Total endo-β-1,3-glucanase (A) and endochitinase (B) activities present in apoplastic extracts from NA and CA, noninfected, and snow-mold-infected (INF) winter rye leaves. Activities are presented as the means ± SE of at least three independent experiments. Both glucanase and chitinase activities were significantly higher in cold-acclimated CA5 plants ($P < 0.01$, $n = 5$) than in nonacclimated NA20 plants.The enzyme activities in snow-mold-infected NA18 INF, NA2 INF, and CA2 INF plants were not significantly different from those in noninfected NA18, NA2, and CA2 plants, respectively.

contrast, the 16-kD polypeptide accumulated to a high level in apoplastic extracts of NA2 and CA2 plants (Fig. 4A, lanes 7 and 5, respectively), where ice crystals grew in the shape of hexagonal columns (Fig. 3E) and hexagonal bipyramids with straight faces (Fig. 3G), which indicated a lower level of antifreeze activity in the leaves. The different forms of ice crystals observed in apoplastic protein extracts obtained from cold-acclimated and snow-mold-infected winter rye may reflect the accumulation of different classes of AFPs. This is supported by Hon et al. (1995), who proposed that the complexity of the faceted surfaces of bipyramidal ice crystals may indicate the presence of multiple AFPs, each of which binds onto a different plane of the ice crystal lattice.

Enzyme Activities

The apoplastic proteins extracted from NA, CA, and snow-mold-infected rye were further characterized by assaying the glucanase and chitinase activities in the apoplastic extracts (Fig. 5). The specific activities of both enzymes, calculated per milligram of apoplastic protein, decreased in response to cold temperature as well as in response to snow mold infection at 18°C (data not shown), because different apoplastic proteins accumulated under these conditions. In terms of the total activities of endoglucanases and endochitinases (calculated per milligram fresh weight), the highest increase in both enzyme activities was observed in apoplastic extracts of CA5 plants, and was significantly ($P < 0.01$) different from that of NA20 plants (Fig. 5). There were no statistically significant increases in total glucanase and chitinase activities induced by snow mold infection of either NA or CA plants (Fig. 5).

DISCUSSION

Snow Mold-Induced PR Proteins in Winter Rye

Snow molds enter cereal leaves either through stomatal openings or directly through the epidermis (Takenaka and Yoshino, 1987). Once inside, these pathogens usually multiply and spread through intercellular spaces. We have shown that winter rye accumulates three classes of apoplastic PR proteins known as glucanases, chitinases, and TLPs in response to snow mold attack (Figs. 2 and 4). The apoplastic location of winter rye PR proteins has been confirmed by immunolocalization in previous studies (Antikainen et al., 1996; Pihakaski-Maunsbach et al., 1996). In addition, measurable endoglucanase and endochitinase activities in apoplastic extracts of snow-mold-infected plants (Fig. 5) suggest that these proteins may inhibit snow mold growth. Mauch et al. (1988) have shown that combinations of glucanases and chitinases are more effective inhibitors of fungal growth than either enzyme alone. Therefore, it is likely that the combination of GLPs, CLPs, and TLPs induced by snow mold penetration of winter rye may provide one component of resistance to the disease.

Cold-Induced Resistance to Snow Molds

Snow molds usually damage plants in areas with deep snow cover. Beneath the snow, photosynthesis is impaired and host plants slowly deplete their carbohydrates and protein reserves. In this weakened state, winter cereals and overwintering grasses are predisposed to disease caused by *M. nivale*, *Typhula* spp., and *S. borealis*. Winter cereal cultivars differ in their resistance to snow mold (Hömmö, 1994a), which is thought to be a nonspecific mechanism related to factors such as plant size, carbohydrate reserves, and low metabolic rates (Gaudet, 1994). Specific snow mold resistance could be based on morphological or biochemical defense mechanisms of cereals, such as the ability to prevent the foliar penetration of the fungus (Hömmö, 1994b). Using the leaf segment test, Hömmö (1994b) found the winter rye cv Musketeer to be one of the most susceptible cultivars to the snow mold *M. nivale*. On the other hand, in the field tests, cv Musketeer showed moderate resistance to snow mold.

We studied the responses of CA Musketeer winter rye to snow mold infection at low temperatures. The plants (CA5 and CA2, Fig. 4) accumulated all three classes of PR proteins in the apoplast during cold acclimation but did not exhibit a significant increase in the total apoplastic protein following snow mold infection at low temperature (Fig. 2). There was also no increase in glucanase and chitinase activities after snow mold infection (Fig. 5). From these results, we suggest that the GLPs, CLPs, and TLPs that accumulate during cold acclimation may provide resistance to pathogens in advance of infection.

Injury caused by freezing can increase the susceptibility of plants to pathogens, although it has been shown that freeze-injured CA barley roots produce lytic and inhibitory substances that inhibit the growth of bacteria (Olien and Smith, 1981). By controlling disease microorganisms, the post-thaw tissue degeneration is restricted to localized freeze injuries so that there is a greater chance of tissue recovery and growth. While the compounds reported by Olien and Smith (1981) have not been isolated, they might have been AFPs. In fact, we have previously shown that CA winter rye roots accumulate CLPs, which may play a role in a defense against soil-borne pathogens (Antikainen et al., 1996).

Antifreeze Activity Associated with PR Proteins

In winter rye, cold-induced apoplastic proteins with similarity to PR proteins have the ability to modify ice (Fig. 3; Hon et al., 1995). The antifreeze activity is high in apoplastic extracts from plants that are exposed to both low temperature and snow molds (Fig. 3). However, when immunologically similar apoplastic proteins are induced by snow mold in winter rye at warm temperatures, the apoplastic extract lacks antifreeze activity (Fig. 3). Therefore, antifreeze activity is induced by low temperatures. These results suggest that different isoforms of PR proteins accumulate in winter rye leaves in response to either pathogens or low temperature. These isoforms may be different gene products or they may result from post-translational modi-

fications of the proteins. The pathogen-induced protein extracts exhibit glucanase and chitinase activities, whereas low-temperature-induced extracts exhibit both enzyme and antifreeze activities. Because the acquisition of antifreeze activity only requires modification of the surface of the protein to form an ice-binding domain (Sicheri and Yang, 1995; Yang et al., 1998), it is more likely that a protein will be altered to confer antifreeze activity rather than modified to introduce a catalytic site. Therefore, it is possible that rye AFPs may have evolved from PR proteins and now play a role in both freezing and pathogen resistance.

The accumulation of extracellular AFPs may explain why conditioning plants to low temperatures increases snow mold resistance. Winter rye cold-acclimated prior to snow mold infection accumulates high amounts of apoplastic GLPs, CLPs, and TLPs with both antifreeze activity and enzymatic activity. Although snow mold infection does not enhance the accumulation of PR proteins or enzymatic activities, the level acquired during cold acclimation may be sufficient to make initial colonization of the plants by pathogens difficult. GLPs, CLPs, and TLPs already present in the apoplast may enable winter rye to respond more rapidly and strongly upon snow mold attack.

Resistance to both freezing and pathogens is a desirable trait for overwintering cereals because freezing, snow molds, and their interaction may decrease plant survival and lead to decreases in yield. Understanding the overlap of function and regulation of AFPs and snow-mold-induced PR proteins may improve our understanding of the relationship between resistance to freezing and resistance to pathogens and our ability to choose multipurpose traits in crop improvement programs.

ACKNOWLEDGMENTS

We thank Mari Helminen for excellent technical assistance with snow mold inoculations and incubations at the Agricultural Research Centre, Jokioinen, Finland, and Dr. Grant McLeod, Agriculture Canada, Swift Current, Saskachawan, Canada, for cv Musketeer rye seeds.

Received February 26, 1999; accepted July 2, 1999.

LITERATURE CITED

Antikainen M, Griffith M (1997) Antifreeze protein accumulation in freezing-tolerant cereals. Physiol Plant **99:** 423–432

Antikainen M, Griffith M, Zhang J, Hon W-C, Yang DSC, Pihakaski-Maunsbach K (1996) Immunolocalization of antifreeze proteins in winter rye leaves, crowns, and roots by tissue printing. Plant Physiol **110:** 845–857

Bernfeld P (1955) Amylases, alpha and beta. Methods Enzymol **1:** 149–158

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:** 248–254

Chun JU, Yu XM, Griffith M (1998) Genetic studies of antifreeze proteins and their correlation with winter survival in wheat. Euphytica **102:** 219–226

Collinge DB, Gregersen PL, Thordal-Christenssen H (1994) The induction of gene expression in response to pathogenic microbes. *In* AS Basra, ed, Mechanisms of Plant Growth and Improved Productivity. Modern Approaches. Marcel Dekker, New York, pp 391–433

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

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 **36:** 712–718

Gaudet DA (1994) Progress towards understanding interactions between cold hardiness and snow mold resistance and development of resistant cultivars. Can J Plant Pathol **16:** 241–246

Gaudet DA, Laroche A (1997) Winter survival of cereals parasitized by snow mold. *In* PH Li, THH Chen, eds, Plant Cold Hardiness. Plenum Press, New York, pp 331–342

Griffith M, Ala P, Yang DSC, Hon WC, 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 freezing-tolerant plants. Adv Low-Temp Biol **3:** 107–139

Griffith M, McIntyre HCH (1993) Interrelationship of growth and frost tolerance in winter rye. Physiol Plant **87:** 335–344

Hiilovaara-Teijo M, Palva ET (1999) Molecular responses in cold-adapted plants. *In* R Margesin, F Schinner, eds, Cold-Adapted Organisms—Ecology, Physiology, Enzymology, Molecular Biology. Springer, Heidelberg, pp 349–384

Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. Calif Agric Exp Stat Circ **347:** 1–32

Hömmö LM (1994a) Hardening of some winter wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.), triticale (× *Triticosecale* Wittmack) and winter barley (*Hordeum vulgare* L.) cultivars during autumn and the final winter survival in Finland. Plant Breed **112:** 285–293

Hömmö LM (1994b) Screening winter rye cultivars for snow mould (*Microdochium nivale*) resistance. Plant Pathol **43:** 740–750

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

Jamalainen EA (1974) Resistance in winter cereals and grasses to low-temperature parasitic fungi. Annu Rev Phytopathol **12:** 281–302

Krol M, Griffith M, Huner NPA (1984) An appropriate physiological control for environmental temperature studies: comparative growth kinetics of winter rye. Can J Bot **62:** 1062–1068

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 PR-proteins are chitinases. Proc Natl Acad Sci USA **84:** 6750–6754

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

Mauch F, Mauch-Mani B, Boller T (1988) Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by the combination of chitinase and β-1,3-glucanase. Plant Physiol **88:** 936–942

Meyer K, Keil M, Naldrett MF (1999) A leucine-rich repeat protein of carrot that exhibits antifreeze activity. FEBS Lett **447:** 171–178

Nakajima T, Abe J (1996) Environmental factors affecting expression of resistance to pink snow mold caused by *Microdochium nivale* in winter wheat. Can J Bot **74:** 1783–1788

Olien CR, Smith MN (1981) Extension of localized freeze injury in barley by acute post-thaw bacterial disease. Cryobiology **18:** 404–409

Pihakaski-Maunsbach K, Griffith M, Antikainen M, Maunsbach A (1996) Immunogold localization of glucanase-like antifreeze protein in cold acclimated winter rye. Protoplasma **191:** 115–125

Sabehat A, Weiss D, Lurie S (1998) Heat-shock proteins andcross-tolerance in plants. Physiol Plant **103:** 437–441

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning. A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

SAS Institute (1985) SAS User's Guide: Statistics, Ed 5. SAS Institute, Cary, NC

Sicheri F, Yang DSC (1995) Ice binding structure and mechanism of an antifreeze protein from winter flounder. Nature **375:** 427–431

Stintzi A, Heitz T, Prasad V, Wiedemann-Merdinoglu S, Kauffmann S, Geoffroy P, Legrand M, Fritig B (1993) Plant pathogenesis-related proteins and their role in defense against pathogens. Biochimie **75:** 687–706

Takenaka S, Yoshino R (1987) Penetration of *Typhula incarnata* in wheat plants differing resistance. Ann Phytopathol Soc Jpn **53:** 566–569

Tamás L, Huttová J, Žigová Z (1997) Accumulation of stress-proteins in intercellular spaces of barley leaves induced by biotic and abiotic factors. Biol Plant **39:** 387–394

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 by frost hardening. *In* Å Kaurin, O Junttila, J Nilsen, eds, Plant Production in the North. Norwegian University Press, Tromsø, Norway, pp 127–133

Tronsmo AM (1993) Resistance to winter stress factors in half-sib families of *Dactylis glomerata*, tested in a controlled environment. Acta Agric Scand **43:** 89–96

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 Defence Responses (abstract). Kluwer Academic Publishers, Dordrecht, The Netherlands, p 369

Worrall D, Elias L, Ashford D, Smallwood M, Sidebottom C, Lillford P, Telford J, Holt C, Bowles D (1998) A carrot leucine-rich-repeat protein that inhibits ice recrystallization. Science **282:** 115–117

Yang DSC, Hon WC, Bubanko S, Xue YQ, Seetharaman J, Hew CL, Sicheri F (1998) Identification of the ice-binding surface on a type III antifreeze protein with a "flatness function" algorithm. Biophys J **74:** 2142–2151

Zhu B, Chen THH, Li PH (1993) Expression of an ABA-responsive osmotin-like gene during induction of freezing-tolerance in *Solanum commersonii*. Plant Mol Biol **21:** 729–735