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β-1,3-Glucanase Is Cryoprotective in Vitro and Is Accumulated in Leaves during Cold Acclimation[1]

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We have used isolated spinach (*Spinacea oleracea* L.) thylakoid membranes to investigate the possible cryoprotective properties of class I β-1,3-glucanase (1,3-β-D-glucan 3-glucanohydrolase; EC 3.2.1.39) and chitinase. Class I β-1,3-glucanase that was purified from tobacco (*Nicotiana tabacum* L.) protected thylakoids against freeze-thaw injury in our in vitro assays, whereas class I chitinase from tobacco had no effect under the same conditions. The β-1,3-glucanase acted by reducing the influx of solutes into the membrane vesicles during freezing and thereby reduced osmotic stress and vesicle rupture during thawing. Western blots probed with antibodies directed against tobacco class I β-1,3-glucanase showed that in spinach and cabbage (*Brassica oleracea* L.) leaves an isoform of 41 kD was accumulated during frost hardening under natural conditions.

Freezing resistance in potentially winter-hardy plant species requires a period of cold acclimation preceding the stress. Numerous biochemical changes are correlated with improved frost tolerance during the hardening period (Levitt, 1980). Some proteins increase in abundance and several cold-induced mRNAs have been cloned (for reviews, see Guy [1990]; Thomashow [1993]). It has yet to be established, however, which of these changes in gene expression are adaptations to growth at the lower temperature and which have a functional role in subsequent resistance to freeze-thaw damage.

Freezing injury is a consequence of the crystallization of water in the apoplastic space (Beck et al., 1984; Pearce and Willison, 1985). It has been shown that several cold-induced proteins accumulate in the apoplast of winter rye (*Secale cereale* L.) leaves and that some of these proteins have thermal hysteresis and recrystallization inhibition activity (Marentes et al., 1993; Hon et al., 1994). Thermal hysteresis or antifreeze proteins have been characterized from many species of fish and terrestrial arthropods (DeVries and Cheng, 1992; Duman et al., 1993; Yeh and Feeney, 1996) and have also been detected in a wide variety of plant species (Griffith et al., 1992; Urrutia et al., 1992; Duman and Olsen, 1993). In rye some of these proteins have been identified as glucanase- or chitinase-like proteins by immunoblotting them with antisera raised against the tobacco (*Nicotiana tabacum* L.) enzymes (Hon et al., 1995; Griffith and Antikainen, 1996). A thermal hysteresis protein from the bittersweet nightshade (*Solanum dulcamara* L.), which has recently been purified, showed no similarities to glucanases or chitinases (Duman, 1994).

The functional role of these various thermal hysteresis proteins in plant frost-hardiness is still unclear, especially since their very low thermal hysteresis activity (typically 0.2–0.5°C; Urrutia et al., 1992; Duman and Olsen, 1993) makes a role as antifreeze proteins, similar to the situation in Arctic and Antarctic fish and insects, highly unlikely. In addition to their thermal hysteresis activity, these proteins could also have direct effects on the stability of cellular membranes. It has recently been shown that a thermal hysteresis protein from a beetle protected isolated gut cells from a centipede during freezing (Tursman and Duman, 1995). Also, fish antifreeze proteins have been shown to stabilize the plasma membrane of some mammalian cells during freezing and low-temperature storage (Rubinsky et al., 1991a, 1991b) and phosphatidylcholine liposomes during a temperature-induced lipid-phase transition (Hays et al., 1996). It has recently been suggested that the thermal hysteresis protein from *S. dulcamara* may stabilize isolated protoplasts from cabbage (*Brassica oleracea*) against freeze-thaw damage (Newton and Duman, 1995). On the other hand, fish antifreeze proteins have been shown to be cryotoxic to spinach (*Spinacea oleracea* L.) thylakoid membranes (Hincha et al., 1993b).

We have developed assays using isolated thylakoid membranes from nonacclimated spinach leaves to identify cryoprotective proteins (for review, see Hincha et al. [1996]). These assays have been used in the characterization and purification of a cold-induced cryoprotective protein (cryoprotectin) from cabbage leaves (Sieg et al., 1996) and in the analysis of the cryoprotective activity of several Gal-specific seed lectins (Hincha et al., 1993a).

The present report deals with the possible role of β-1,3-glucanases in plant cold acclimation. At least three struc-

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Abbreviation: PR, pathogenesis-related.

tural classes of β-1,3-glucanases have been identified: The class I isoforms are basic proteins localized in the cell vacuole extension (Shinshi et al., 1988; Keefe et al., 1990). The class II and III isoforms are acidic proteins secreted into the extracellular space. Included in this group are the PR proteins PR-2, PR-N, PR-O, and PR-Q' (Kauffmann et al., 1987; Payne et al., 1990) and two glycoproteins localized in the style of tobacco flowers (Lotan et al., 1989; Ori et al., 1990). Finally, a distinct, intracellular class of "ersatz" β-1,3-glucanases is induced by a virus infection in class I β-1,3-glucanase-deficient mutants of *Nicotiana sylvestris* and *N. tabacum* as a substitute for the β-1,3-glucanase induced in wild-type plants (Beffa et al., 1993). β-1,3-Glucanases have been implicated in several physiological and developmental processes, including cell division, pollen formation, and seed germination, and in plant responses to ozone (Waterkeyn, 1967; Worrall et al., 1992; Vögeli-Lange et al., 1994; Schrauder et al., 1996). They also appear to be part of both the constitutive and induced defense against pathogenic fungi (Mauch et al., 1988; Mauch and Staehelin, 1989; Keefe et al., 1990; Zhu et al., 1994). Recent evidence suggests that they also have an important role in viral pathogenesis (Beffa et al., 1996). Here we show that class I β-1,3-glucanase has potent cryoprotective activity in vitro and that β-1,3-glucanases are accumulated in spinach and cabbage leaves during cold acclimation.

MATERIALS AND METHODS

For nonacclimated plant material, spinach (*Spinacea oleracea* L. cv Monnopa) and cabbage (*Brassica oleracea* L. cv Grüfiwi) were grown in a growth chamber under nonhardening conditions (Sieg et al., 1996). For cold-acclimated material, the plants were grown in a garden plot. They were exposed to fall and winter conditions and during that time cold-hardened under natural conditions. These cold-acclimated plants were harvested between November and February after at least 5 d with subzero temperatures. The tissue used to isolate class I chitinase and class I β-1,3-glucanase was the cloned line 275N of tobacco (*Nicotiana tabacum* L. cv Havana 425) pith parenchyma tissue cultured as described previously (Eichholz et al., 1983).

Measurement of Chitinase and β-1,3-Glucanase Activity

Chitinase and β-1,3-glucanase activities were measured radiometrically using [^{3}H]acetyl-chitin or [^{3}H]laminarin, respectively, as substrates (Boller et al., 1983; Keefe et al., 1990).

Assay for Cryoprotective Activity

The ability of the different proteins to maintain thylakoid membrane integrity during a freeze-thaw cycle was measured as the preservation of thylakoid volume (Hincha and Schmitt, 1992; Sieg et al., 1996). The membranes were isolated from nonhardy spinach leaves and washed three times either in 5 mM NaCl for volume measurements or in 10 mM $MgCl_2$ and 20 mM K_2SO_4 for plastocyanin release measurements (Hincha and Schmitt, 1988). Samples (0.4 mL with thylakoids equivalent to 0.5 mg chlorophyll mL^{-1}) containing different amounts of protein were frozen for 3 h at −20°C. Samples were placed in a freezer in air at this temperature. Nucleation occurred spontaneously. The samples needed approximately 30 min to reach −20°C. They were thawed rapidly (within 2–3 min) in a water bath at room temperature. After thawing, samples were diluted with an equal volume of 10 mM $MgCl_2$ and the packed thylakoid volume was measured after hematocrit centrifugation. Protection was calculated relative to control samples that were incubated for the same time at −20°C (0% protection) or 0°C (100% protection) in the absence of added protein. Alternatively, freeze-thaw damage was measured as the release of the soluble, lumenal protein plastocyanin (Hincha et al., 1985; Hincha and Schmitt, 1995). After thawing the membranes were sedimented by centrifugation (15 min at 16,000*g*), and plastocyanin in the supernatants was determined by single radial immunodiffusion, as described previously (Hincha and Schmitt, 1988, 1995).

Purification of Tobacco Class I β-1,3-Glucanase and Chitinase

The enzymes were purified from tobacco tissue by a modification of the methods described previously (Eichholz et al., 1983). All operations were performed on ice or at 6°C using buffers supplemented with 10 mM β-mercaptoethanol and 1 mM EDTA. Tissue harvested 4 weeks after subculture was homogenized in 3 volumes of 10 mM sodium ascorbate (pH 5.0) for 1 min at maximum speed in an Omnimixer (Sorvall). The homogenate was centrifuged for 30 min at 10,000*g*, and the resultant pellet was extracted twice by stirring for 30 min in one-third of the original volume of 0.5 M sodium ascorbate (pH 5.0) and centrifuged as above. The extracts were pooled, dialyzed against 10 volumes of 100 mM sodium acetate (pH 5.0), and then diluted with an equal volume of water. The diluted extract was applied to a column (2.5 × 20 cm, CM-Sephadex C50, Pharmacia) equilibrated in 10 mM sodium acetate (pH 5.0). The column was eluted with a linear gradient of NaCl (0–750 mM) in the same buffer. Chitinase activity was eluted from the column between 40 and 100 mM NaCl, whereas the β-1,3-glucanase activity remained on the column and was eluted with 2 M NaCl in the buffer. The active fractions were concentrated to approximately 20 mL by dialysis against 30% (w/v) PEG 6000 in 10 mM Tris-HCl (pH 7.5), dialyzed against the same buffer, and applied to a column (1.4 × 7.5 cm, DEAE-Sephacel, Pharmacia) equilibrated in 10 mM Tris-HCl (pH 7.5). The column was eluted with the same buffer and fractions containing the enzymes were pooled. The protein was concentrated by dialysis against 30% (w/v) PEG 6000 in 20 mM NH_4HCO_3 and stored at −20°C. β-1,3-Glucanase and chitinase, consisting of a mixture of the 34-kD A-isoform and the 32-kD B-isoforms (Shinshi et al., 1987), were >95% pure as judged by SDS-PAGE.

The purity of the tobacco β-1,3-glucanase is documented in Figure 1. The gel shows a single protein band over a 20-fold range of applied protein, from 0.5 to 10 μg. At the highest concentration used, where the gel has been heavily

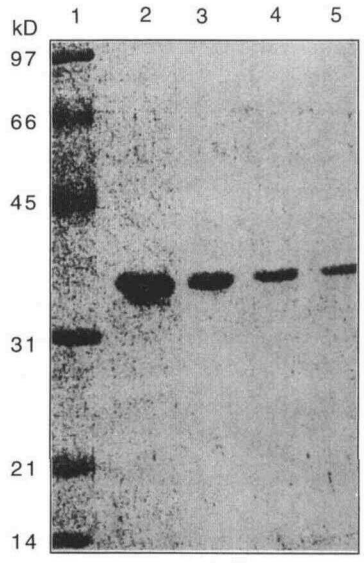

Figure 1. Analysis of β-1,3-glucanase purified from tobacco cell culture by SDS-PAGE and Coomassie blue staining. Lane 1 shows standard proteins. Lanes 2 to 5 show purified tobacco β-1,3-glucanase. The amounts of protein applied to the gel were 10 μg (lane 2), 2.5 μg (lane 3), 1 μg (lane 4), and 0.5 μg (lane 5).

overloaded, some protein of a slightly lower molecular mass becomes visible. This is due to small amounts of the degradation products of the β-1,3-glucanase, because it is recognized in western blots by an antibody specific for β-1,3-glucanase (data not shown).

Gel Electrophoresis and Western Blotting

Crude extracts were obtained by grinding 5 g of leaves from spinach or cabbage plants in 10 mL of an ice-cold buffer (50 mM Tris-acetate [pH 7.8] and 2% [w/v] Polyclar AT [insoluble PVP, Serva, Heidelberg, Germany]) with a mortar and pestle. Homogenates were clarified by centrifugation (15 min at 16,000*g*), mixed with an equal volume of SDS sample buffer (Laemmli, 1970), and boiled for 5 min. Samples were subjected to electrophoresis in the presence of SDS in 12.5% polyacrylamide gels. The separated proteins were transferred to nitrocellulose membranes by electroblotting (Towbin et al., 1979). Unoccupied binding sites on the membranes were blocked by incubation in 3% (w/v) milk powder in 25 mM Tris and 150 mM NaCl (pH 7.5; Johnson et al., 1984). Filters were probed with rabbit anti-tobacco class I β-1,3-glucanase IgG (Felix and Meins, 1985). Bound IgG on the filters was visualized with a goat anti-rabbit serum labeled with horseradish peroxidase (Bio-Rad) as described by Sieg et al. (1996).

Determination of Protein and Chlorophyll

Protein was measured in the samples after boiling in electrophoresis sample buffer by amido black staining (Sheffield et al., 1987) using the dye elution conditions of Schaffner and Weissmann (1973). BSA was used as a standard. Chlorophyll concentrations were determined according to the method of Arnon (1949).

RESULTS

Cryoprotective Activity of Tobacco β-1,3-Glucanase

It has recently been shown that one of the cold-induced thermal hysteresis proteins in winter rye has β-1,3-glucanase activity (Hon et al., 1995). Antibodies raised against the tobacco basic β-1,3-glucanase reacted with the cold-induced rye polypeptides of approximately 33 kD (Griffith and Antikainen, 1996). Therefore, these proteins must share extensive structural similarities with the pathogen-induced basic β-1,3-glucanase from tobacco. Since none of the cold-induced glucanases is available in sufficient purity and quantity, we decided to conduct our in vitro studies of possible cryoprotective effects of β-1,3-glucanases with the structurally similar tobacco enzyme.

Photosynthetic membranes injured by freezing collapse to a low volume. The effect of β-1,3-glucanase on freeze-induced collapse was assayed by measuring thylakoid volume after a freeze-thaw cycle in the presence of increasing concentrations of protein. The results shown in Figure 2 are expressed as percentages of cryoprotection, i.e. thylakoid volume after freezing relative to thylakoids kept at 0°C (compare with Sieg et al., 1996). Cryoprotection increased linearly with β-1,3-glucanase concentration. No cryoprotection was observed with BSA or with purified tobacco class I chitinase over the same concentration range, indicating that the protection obtained with β-1,3-glucanase is specific for this protein. On a molar basis, tobacco β-1,3-glucanase was 2000 to 3000 times more effective as a cryoprotectant than Suc for isolated spinach thylakoids. Approximately 100 mM Suc was required to achieve 100% cryoprotection under our experimental conditions (compare with Hincha et al., 1989).

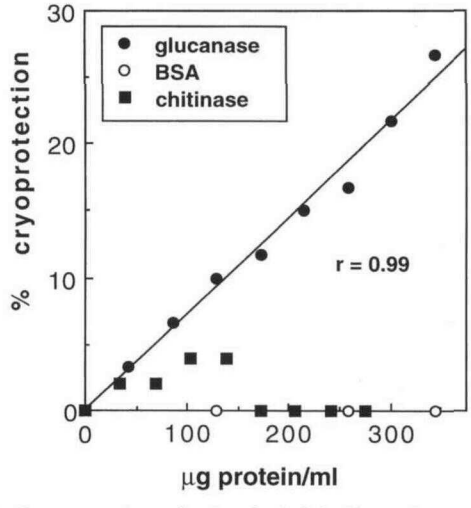

Figure 2. Cryoprotection of spinach thylakoids against mechanical freeze-thaw damage by isolated tobacco class I β-1,3-glucanase, tobacco class I chitinase, and BSA. Thylakoids were isolated from nonhardy spinach leaves and washed in 5 mM NaCl. The samples contained 2.5 mM NaCl and 5 mM Suc in addition to the protein concentrations indicated. After a freeze-thaw cycle, cryoprotective activity of the proteins was determined as preservation of thylakoid volume. The straight line was fitted to the data by linear regression analysis.

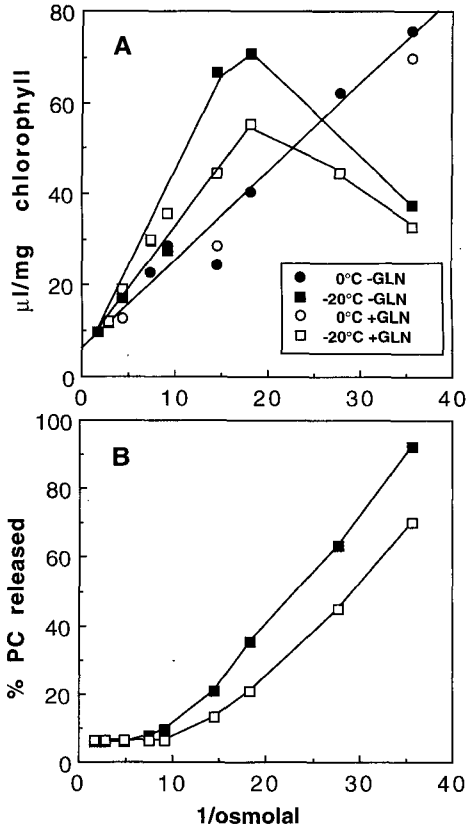

Figure 3. Boyle van't Hoff-type analysis of the freeze-thaw behavior of thylakoid membranes. Thylakoids were washed in 5 mM NaCl. All samples contained 2.5 mM NaCl and 20 to 500 mM Suc. Samples were incubated at either −20 or 0°C for 3 h in the absence (−GLN) or presence (+GLN) of 150 µg tobacco class I β-1,3-glucanase mL^{-1}. A, Effects of a freeze-thaw cycle on thylakoid volume. Volume is plotted as a function of the reciprocal osmolality of the Suc solutions used to suspend the membranes. The data points are the means of two measurements. The straight lines from frozen-thawed samples at low reciprocal osmolalities were fitted to the data by linear regression analysis. +GLN: slope 2.66, $r = 0.99$; −GLN: slope 4.53, $r = 0.99$. Values from 0°C controls in the presence or absence of protein fell on the same regression line (slope 1.80, $r = 0.98$). B, From aliquots of the samples, the membranes were removed by centrifugation and plastocyanin (PC) was determined in the supernatants by immunodiffusion.

To obtain information about the mechanism by which β-1,3-glucanase protected the membranes from freezing damage, thylakoids were incubated for 3 h at 0 or −20°C in solutions containing 20 to 500 mM Suc. The treatments were carried out with and without added β-1,3-glucanase. After the samples were thawed, the volume of the membrane vesicles and plastocyanin release were measured. The results of the volume measurements, shown in Figure 3, are expressed in a Boyle-van't Hoff plot of packed thylakoid volume plotted as a function of the reciprocal osmolality of the incubation solutions. In the control experiment thylakoids maintained at 0°C behaved as ideal osmometers: volume increased linearly with reciprocal osmolality and no differences were found in the absence or presence of added β-1,3-glucanase (Fig. 3A). After freezing and thawing, thylakoids suspended in moderate to high Suc concentrations expanded to larger volumes than unfrozen controls, indicating a solute influx during freezing. Low solute concentrations (1/osmol > 10; concentration < 100 mM) do not give the vesicles enough osmotic support after thawing. Therefore, under these conditions the vesicles rupture, losing plastocyanin (Fig. 3B), and collapse to a low volume (Fig. 3A). The expansion of thylakoids due to solute loading during freezing was reduced in the presence of β-1,3-glucanase (Fig. 3A). It also reduced rupture of the membrane vesicles, as indicated by a reduced release of plastocyanin (Fig. 3B).

The time course of freeze-thaw damage with and without β-1,3-glucanase was determined for isolated thylakoids incubated at 0 or −20°C in a medium comprising the major groups of solutes found in the chloroplast stroma (Hincha and Schmitt, 1988). Tobacco β-1,3-glucanase did not affect the time-dependent release of plastocyanin from membranes at 0°C (Fig. 4). The frozen-thawed membranes showed a rapid initial release of plastocyanin, followed by a slow phase of release. The presence of β-1,3-glucanase decreased the slow release but had no effect on the rapid initial loss of plastocyanin.

Cold Acclimation and the Accumulation of β-1,3-Glucanase

Since isolated tobacco β-1,3-glucanase proved to be an efficient cryoprotectant for thylakoids isolated from nonacclimated spinach, we were interested in determining

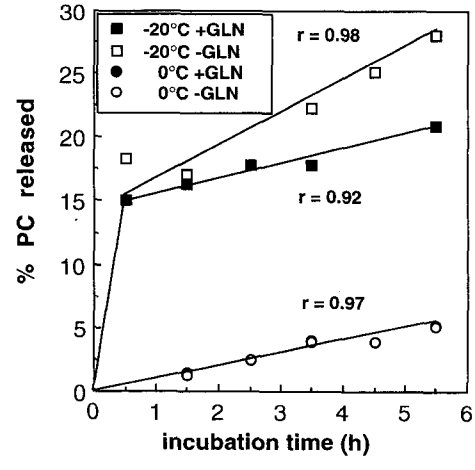

Figure 4. Effect of tobacco class I β-1,3-glucanase on plastocyanin (PC) release from spinach thylakoids in the presence of an artificial stroma medium. Thylakoids were washed in 10 mM $MgCl_2$ and 20 mM K_2SO_4 and incubated in 5 mM $MgCl_2$, 10 mM K_2SO_4, 150 mM potassium glutamate, and 52.5 mM Suc in the absence (−GLN) or presence (+GLN) of 150 µg β-1,3-glucanase mL^{-1} for the times indicated, either at −20 or 0°C. Samples incubated at −20°C were transferred to a freezer at time 0. For a better comparison, the amount of plastocyanin released from the thylakoids directly after transfer from the washing medium to the incubation medium was subtracted from the amounts released at all of the other times. All straight lines were fitted to the data by linear regression analysis (−20°C/−GLN, slope 2.12; −20°C/+GLN, slope 1.12; and 0°C/±GLN, slope 0.92).

whether a similar protein also accumulated in leaves during cold acclimation. Total soluble protein was extracted from both cold-acclimated and nonacclimated spinach and cabbage leaves and was fractionated by SDS-PAGE. The polypeptides were then transferred onto nitrocellulose membranes and the resulting blots were probed with a monospecific antibody directed against tobacco class I β-1,3-glucanase (Felix and Meins, 1985), which also cross-reacts with the class II and III isoforms (Beffa et al., 1993).

Figure 5 shows that a single major band corresponding to an apparent molecular mass of 41 kD was detected in extracts prepared from both cold-acclimated and nonacclimated cabbage leaves (lanes 1 and 2). A major band corresponding to approximately the same molecular mass was also found in the extracts of cold-acclimated spinach leaves (lane 3), whereas a doublet of 38 and 35 kD was found in the extracts of nonacclimated spinach. This indicates that the extracts contained polypeptides serologically related to tobacco β-1,3-glucanase but larger in molecular mass than the 33-kD tobacco class I β-1,3-glucanase that was used as a standard (lane 5). The important observation, however, is that for both cabbage and spinach the immunoreactive polypeptide was more abundant in the cold-acclimated leaf tissue.

DISCUSSION

We have investigated the possible role of β-1,3-glucanases in the response of plants to low temperatures. The most important finding was that tobacco class I β-1,3-glucanase has pronounced cryoprotective activity in the in vitro thylakoid assays (Figs. 2–4). It was also interesting that this appears not to be a general effect of stress-induced PR proteins. Class I chitinases, which are coordinately induced with β-1,3-glucanases in response to ozone (Schraudner et al., 1992) and pathogen infection (for review, see Meins et al. [1992]), were not effective (Fig. 2).

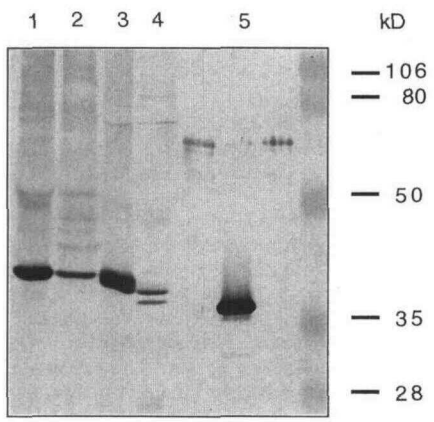

Figure 5. Western blot of crude leaf extracts. The blot was probed with antibodies raised against tobacco class I β-1,3-glucanase. The molecular mass of the standard proteins is given in kilodaltons. Twenty micrograms of soluble protein from the leaves of cold-acclimated cabbage (lane 1), nonacclimated cabbage (lane 2), cold-acclimated spinach (lane 3), and nonacclimated spinach (lane 4) was loaded in each lane. Lane 5 shows purified tobacco class I β-1,3-glucanase (0.15 μg).

Functionally, the cryoprotective effect of the β-1,3-glucanase was similar to that of other proteins with cryoprotective activity, for which specific interactions with the membranes during freezing have been implicated. The tobacco enzyme prevented vesicle rupture, as shown by thylakoid volume measurements. This is the result of a decreased solute loading of the vesicles during freezing (Fig. 3A), which reduces osmotic stress and rupture during thawing. A similar effect on the solute influx into thylakoids during freezing has been described for several lectins (Hincha et al., 1993a) and for cryoprotectin (Hincha et al., 1990). Only cryoprotectin has been found to reduce the amount of plastocyanin lost from thylakoids during the rapid phase of freeze-thaw damage (Hincha et al., 1990). The lectins (Hincha et al., 1993a) and β-1,3-glucanase (Fig. 4) had no effect on this part of the freezing injury.

Solute loading also takes place in the absence of freezing (Hincha, 1986) and leads to slow time-dependent rupture at 0°C (Hincha and Schmitt, 1988). Cryoprotectin and β-1,3-glucanase had no effect on thylakoid rupture in an unfrozen solution (Fig. 4; Hincha et al., 1990), presumably because the protective proteins have to be concentrated in the vicinity of the membranes by the crystallization of ice. Cryoprotective lectins, on the other hand, have been shown to reduce the solute permeability of thylakoids already in the absence of freezing (Hincha et al., 1993a, 1997), leading to a reduction of time-dependent rupture at 0°C. This may indicate differences in the modes of interaction of the different proteins with thylakoid membranes.

The structural requirements for a protein to prevent freeze-thaw damage to a membrane are not known. Cryoprotectin is different from β-1,3-glucanase, since it lacks glucanase activity and showed no cross-reaction in western blots with antibodies directed against the tobacco β-1,3-glucanase (data not shown). In addition, cryoprotectin has a molecular mass of 7 kD and, on a molar basis, 3 orders of magnitude less protein was necessary to achieve the same degree of cryoprotection as with the class I β-1,3-glucanase from tobacco (Hincha et al., 1996; Sieg et al., 1996). Cryoprotectin is also stable to boiling (Hincha and Schmitt, 1992), whereas glucanases are heat-labile (Moore and Stone, 1972; Wong and Maclachlan, 1979).

In addition to showing cryoprotective activity in vitro, β-1,3-glucanase also accumulates in leaves during cold acclimation. In both spinach and cabbage a 41-kD isoform was strongly induced during cold acclimation under natural conditions (Fig. 5). Thus, it appears that the β-1,3-glucanases identified in cold-acclimated cabbage and spinach leaves (Fig. 5) may be similar to the stylar class II β-1,3-glucanases of tobacco, which are also 41-kD proteins. Whether they have similar primary structures remains to be determined from sequence comparisons. Also, the cabbage and spinach glucanases that accumulate during cold acclimation will have to be purified to homogeneity to ascertain their cryoprotective activity.

We have shown that β-1,3-glucanase from tobacco is cryoprotective for thylakoids, but since the class I β-1,3-glucanase is a vacuolar enzyme (Keefe et al., 1990), this would not be the target membrane of the protein in the

intact tissue. At present, we have no information about whether the tonoplast would also be protected from freezing damage by this protein. Likewise, the cellular localization of the cold-induced, 41-kD β-1,3-glucanase will have to be determined to obtain information about the possible target membrane(s) of this protein.

Another interesting aspect of this study is the fact that the analysis of cold-induced genes and proteins from spinach has not identified any β-1,3-glucanases (for review, see Guy [1990]), although we found a strong accumulation of this protein (Fig. 5). A control experiment revealed that the 41-kD β-1,3-glucanase was not induced in spinach during cold acclimation at 4°C for up to 14 d. Western blots showed only the two weak bands (Figure 5) for nonacclimated spinach, with no detectable change during cold acclimation (data not shown). It therefore seems likely that the 41-kD β-1,3-glucanase is induced by lower temperatures or by the actual freezing of the leaf tissue and the concomitant cellular dehydration, as occurred in the field before the leaves were harvested.

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