Cabbage Cryoprotectin Is a Member of the Nonspecific Plant Lipid Transfer Protein Gene Family[1]

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We have recently purified a protein (cryoprotectin) from the leaves of cold-acclimated cabbage (*Brassica oleracea*) to electrophoretic homogeneity, which protects thylakoids isolated from the leaves of nonacclimated spinach (*Spinacia oleracea*) from freeze-thaw damage. Sequencing of cryoprotectin showed the presence of at least three isoforms of WAX9 proteins, which belong to the class of nonspecific lipid transfer proteins. Antibodies raised against two synthetic peptides derived from the WAX9 proteins recognized a band of approximately 10 kD in western blots of crude cryoprotectin preparations. This protein and the cryoprotective activity could be precipitated from solution by the antiserum. We show further that cryoprotectin is structurally and functionally different from WAX9 isolated from the surface wax of cabbage leaves. WAX9 has lipid transfer activity for phosphatidylcholine, but no cryoprotective activity. Cryoprotectin, on the other hand, has cryoprotective, but no lipid transfer activity. The cryoprotective activity of cryoprotectin was strictly dependent on Ca^{2+} and Mn^{2+} and could be inhibited by chelating agents, whereas the lipid transfer activity of WAX9 was higher in the presence of ethylenediaminetetraacetate than in the presence of Ca^{2+} and Mn^{2+}.

There is great variability in the freezing tolerance (i.e. the temperature at which cellular damage occurs during freezing) of different plant species. In addition, many plants are able to increase their freezing tolerance during a cold acclimation period at a low, but nonfreezing temperature (Levitt, 1980; Steponkus, 1984). Despite intensive research, the molecular basis of constitutive freezing tolerance and cold acclimation capacity remains largely unknown. It has, however, been shown that cold acclimation is accompanied by distinct changes in gene expression. A considerable number of cold-induced genes have been cloned and sequenced (for review, see Guy, 1990; Hughes and Dunn, 1996; Thomashow, 1999). Many of the deduced proteins show no homology to proteins with known functions. Nevertheless, the induction of an array of these proteins in transgenic plants constitutively expressing cold-regulated transcription factors proved the vital role of at least some of these proteins in plant freezing tolerance (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999).

Because membranes are the primary targets of freezing injury in plants (for review, see Steponkus, 1984; Krause et al., 1988; Hincha and Schmitt, 1992b), increased freezing tolerance during cold acclimation should include the protection of membranes during a freeze-thaw cycle. This has been well established for the plasma membrane and chloroplast thylakoid membranes. However, in both cases it is evident that changes in membrane properties alone are not sufficient to account for the effects of cold acclimation on freezing tolerance in vivo (Hincha et al., 1996; Uemura and Steponkus, 1997). Therefore, soluble cryoprotectants are thought to play an important role in the stabilization of cellular membranes during freezing. The involvement of a soluble cold regulated protein from Arabidopsis, COR15am, in stabilizing the plasma membrane and chloroplasts of leaf cells, has been established in transgenic Arabidopsis plants, which constitutively express the appropriate gene (Artus et al., 1996; Steponkus et al., 1998).

Using an in vitro test system we have previously shown that several Gal-binding seed (Hincha et al., 1993) and leaf lectins (Hincha et al., 1997c), and a

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class I β-1,3-glucanase (Hincha et al., 1997b) have cryoprotective activity for isolated thylakoid membranes. We have also purified a cryoprotective protein (cryoprotectin) from the leaves of cold-acclimated cabbage (*Brassica oleracea*). This is a small, boiling-stable protein that could not be purified from the leaves of nonacclimated plants, indicating that cryoprotectin may be cold induced (Sieg et al., 1996).

RESULTS

We have partially sequenced cryoprotectin from preparations that were homogeneous as judged from silver stained SDS-PAGE gels (compare Sieg et al., 1996). The amino terminus was not blocked and we determined the sequences of two distinct N-termini. A further 21 internal peptides were purified after tryptic digestion of cryoprotectin and were also sequenced. Figure 1 shows a synopsis of all sequences where overlapping sequences were combined into continuous sequences. A computer search of the EMBL sequence database showed that all sequences belong to nonspecific lipid transfer proteins (LTPs). The greatest degree of identity was found to different members of the WAX9 family of lipid transfer proteins from broccoli (*Brassica oleracea*). Broccoli and

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           1         10        20         30        40        50
WAX9 A     MAGVMKLACLVLACMIVAGPITANR ALTCGTVNSNVAPCIGYITQGGT-LPGACCTGVS
WAX9 B     MAGLVKLSCLVLACMIVAGPIATNA ALSCGTVSGNLAACIGYLTQNGP-LPRGCCTGVT
WAX9 C     MAGLMKLACLVLACMIVAGPITSKA ALSCGTVNTNVAACIGYLTVG-A-LPRACCTGVS
WAX9 D     MAGLMKLACLIFACMIVAGPITSNA ALSCGTVSGYVAPCIGYLAQNAPAVPTACCSGVT
WAX9 E     MAGVMKLACLVLACMIVAGPITANA ALTCGTVNSNVAPCIGYITQGGP-LPRACCTGVS
WAX9 D                               ALSCGTVSGYVAPCIGYLAQNAPAVPR
WAX9 A/E                             ALTCGTVNSNVAPCIGYITQ
WAX9 D                                                           ACCTGVT

           60        70        80        90        100       110       120
WAX9 A     KLNSMARTTPDRQQACRCLETAARALGPNLNAGRAAGIPKACGVSVPFPISTNT---NCNNVK
WAX9 B     NLNNMARTTPDRQQACRCLVGAANSF-PTLNAARAAGLPKACGVNIPYKISKST---NCNSVR
WAX9 C     KLNSIARTTPDRQQACRCLKTAASALGSGLNAGRAAGLPKACGVNVPFPISLLTRCINCNSVK
WAX9 D     SLNNMARTTPDRQQACRCLVGAANAL-PTINVARAAGLPKACGVNIPYKISKTT---NCNSVK
WAX9 E     KLNSMARTTPDRQQACRCLKTAASALGPNLNAGRAAGIPKACGVSVPFPISTNT---NCNNVK
WAX9 D     SLNN
WAX9 E                        TAASALGPNLNAGR
WAX9 B                     CLVGAANSF-PTLNAAR
WAX9 B/C/D                                  AAGLPK
WAX9 B/D                                              ACGVNIPYK
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Figure 1. Amino acid sequences of four nonspecific LTPs from broccoli (WAX9 A, B, C, and D; Pyee and Kolattukudy, 1995) and one deduced from the sequence of a cDNA cloned from cabbage (WAX9 E). The putative signal sequences (amino acids 1–25) are set off from the start of the mature proteins by a space. In WAX9 E the amino terminal sequence determined from the isolated protein is underlined. The sequences that were used to generate peptides to raise antibodies are doubly underlined (WAX9 A/E for peptide 1 and WAX9 B/D for peptide 2). The sequences determined from an electrophoretically pure preparation of cryoprotectin, together with the assignment to different WAX9 isoforms (bold letters), are shown below the complete sequences. Not all peptides could be unambiguously assigned to one isoform. Dashes were introduced in the sequences for optimal alignment.

cabbage are different varieties of the same species. The sequenced peptides could be completely aligned with the proteins WAX9 B, D, and E. An unambiguous assignment was not possible for the two short C-terminal peptides, but it seems reasonable to assume that they belong to WAX9 B.

We cloned five *wax9* genes from cabbage using PCR-based cloning techniques with primers based on the published sequences of the cDNAs *wax9 A* through *D* from broccoli (Pyee and Kolattukudy, 1995). The forward primer was chosen from the putative signal sequences, as they were the most homologous between the different *wax9* genes. The reverse primer was constructed from the sequences of the mature proteins, which also showed extensive homology. The protein WAX9 E in Figure 1 was deduced from the gene *wax9 E* (accession no. AF093751), a novel member of this gene family.

In accordance with the known localization of the WAX9 proteins in the cuticular wax layer of leaves (Pyee et al., 1994; Pyee and Kolattukudy, 1995), all cloned *wax9* genes have a 25-amino acid amino terminal extension (Fig. 1) that is thought to function as a sorting signal for the secretory pathway. A similar export signal has been found in all cloned LTP genes (Kader, 1996). The mature WAX9 proteins all start with Ala-26 and have a calculated molecular mass of about 9 kD (Pyee et al., 1994). Like all other plant LTPs, the WAX9 proteins have eight conserved cystein residues, six of which were also present in the sequences from cryoprotectin (Fig. 1).

Figure 1 shows that the cryoprotectin preparations used for sequencing, although electrophoretically homogeneous (Sieg et al., 1996), still contained at least three different polypeptides. It therefore seemed possible that cryoprotective activity might be associated with an additional undetected protein. To check this possibility we raised antibodies against two synthetic peptides derived from WAX9 sequences (Fig. 1). In the following discussion the peptide corresponding to the amino terminus of WAX9 A/E is termed peptide 1, and the peptide derived from the internal sequences of WAX9 B/D is termed peptide 2 (compare Fig. 1, doubly underlined sequences). Because we used synthetic peptides there was no danger of cross-contamination from other plant proteins. Figure 2 (lanes 4 and 5) shows that the antiserum reacted much more strongly with peptide 2 than with peptide 1. The serum also detected a band of approximately 10 kD in western blots of cryoprotectin after ammonium sulfate precipitation (compare "Materials and Methods"). A preimmune serum did not react with the peptides or any cabbage proteins on such blots (data not shown).

The antiserum was able to precipitate the 10-kD polypeptide from solution (Fig. 2, compare lanes 1 and 6). Immunoprecipitation could be inhibited by competition with peptide 2, but was hardly inhibited by peptide 1 (Fig. 2, lanes 2 and 3). A control antiserum did not precipitate the protein in the absence or presence of peptide 2 (Fig. 2, lanes 7 and 8).

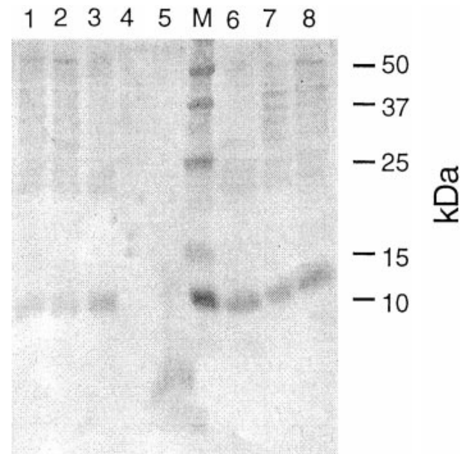

Figure 2. Western-blot analysis of cryoprotectin. After ammonium sulfate precipitation (compare "Materials and Methods"), the proteins were transferred into 10 mM Suc, 1 mM $MnCl_2$, and 1 mM $CaCl_2$ by gel filtration chromatography. Antibodies raised against synthetic peptides derived from WAX9 sequences (compare Fig. 1) were bound to protein A immobilized on magnetic beads. These beads were then used to immunoprecipitate cryoprotectin from solution. In some cases the peptides used for immunization were added to the cryoprotectin solution as specific competitors for antibody binding sites. An antiserum against Rubisco was used as an unspecific control. The supernatants were analyzed on western blots probed with the same antiserum. Lane 1, immunoprecipitation with anti-WAX9 antiserum; lane 2, as lane 1, but competition with peptide 1; lane 3, as lane 1, but competition with peptide 2; lane 4, peptide 1; lane 5, peptide 2, M, marker proteins; lane 6, cryoprotectin before immunoprecipitation; lane 7, immunoprecipitation with anti-Rubisco antiserum; lane 8, as lane 7, but competition with peptide 2.

When the supernatants after immunoprecipitation were assayed for cryoprotective activity it was found that the antiserum also removed the cryoprotective activity from solution (Fig. 3, compare samples 3 and 4). Again, the effect of the antiserum could be competitively inhibited by peptide 2, but not by peptide 1, and the control antiserum showed no effect. This result was obtained irrespective of whether cryoprotective activity was determined from thylakoid volume measurements (Fig. 3, bar graph) or from plastocyanin release measurements (Fig. 3, western blot). We take the data presented in Figures 1 through 3 as unequivocal evidence that the cryoprotective activity is associated with one or more WAX9-like proteins.

There were no published data on the lipid transfer activity of WAX9, which was inferred to be an LTP from the sequence similarity to other LTPs (Pyee and Kolattukudy, 1995). The fact that the amino acid sequences determined for cryoprotectin were identical to those of WAX9 proteins gave rise to two important questions: do cryoprotectin and WAX9 have lipid transfer activity, and does WAX9 (and possibly other LTPs) have cryoprotective activity?

The protocols for the isolation of cryoprotectin and WAX9 use harsh chemical treatments. The isolation

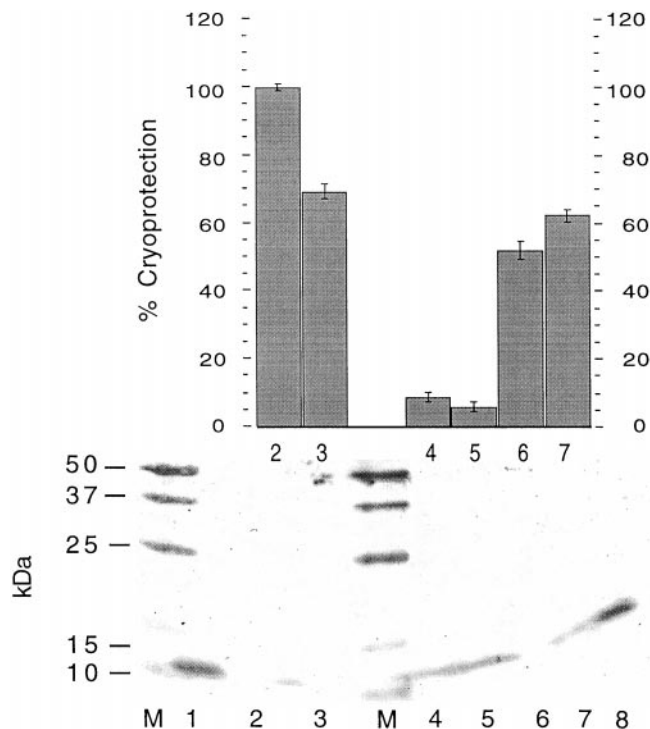

Figure 3. Cryoprotective activity of the supernatants of cryoprotectin preparations after immunoprecipitation (see Fig. 2 for details). Cryoprotection was measured with the hematocrit centrifugation assay (bar graph) and as plastocyanin release (western blot). The data in the bar graph are the means ± SEM from the results of three to four experiments with a total of six to eight samples. The samples: 1 and 8, sonicated thylakoids; 2, unfrozen control thylakoids; 3, thylakoids frozen with cryoprotectin; 4, thylakoids frozen with cryoprotectin after immunoprecipitation with anti-WAX9 antibodies; 5, as 4, but competition with peptide 1; 6, as 4, but competition with peptide 2; and 7, as 4, but immunoprecipitation with anti-Rubisco antiserum. M, Marker proteins.

of WAX9 involves its solubilization from the cuticular wax layer with chloroform, and the purification of cryoprotectin involves the exposure of the protein to β-mercaptoethanol or urea. We therefore tested whether these potentially denaturing substances have an effect on the cryoprotective activity of cryoprotectin or on the lipid transfer activity of WAX9. Figure 4A shows that even after treatment with 300 mM β-mercaptoethanol or 6 M urea, or after partitioning against chloroform, cryoprotective activity was not diminished by more than 20%, compared with untreated control samples. WAX9 clearly showed lipid transfer activity and this was only weakly inhibited by the same treatments (Fig. 4B).

Both proteins were subjected to gel filtration chromatography using a matrix designed for the separation of peptides and small proteins in accordance with their expected molecular weights. Cryoprotectin in these crude preparations had to be pretreated with 6 M urea to dissolve large aggregates (data not shown). In the fractions obtained by gel filtration chromatography of WAX9 (Fig. 5A) and cryoprotectin (Fig. 5B), cryoprotective activity and lipid transfer activity were determined. WAX9 eluted in a single peak, as determined by lipid transfer measurements, but none of the fractions had cryoprotective activity. The results obtained with the crude cryoprotectin sample were less clear. The majority of the cryoprotective activity eluted in one peak, with a smaller apparent molecular mass (8.4 kD) than WAX9 (11.5 kD). However, the same fractions also contained lipid transfer activity, which eluted in a much broader peak that also contained proteins of higher apparent molecular mass.

To separate the cryoprotective and lipid transfer activities, the fractions comprising the highest cryoprotective activity (from 84–93 mL elution volume in Fig. 5B) were pooled and applied to a reverse-phase column. To ensure that lipid transfer activity was not inactivated under the conditions employed in reverse-phase chromatography, WAX9 was fractionated under the same conditions. To avoid excessive dilution of WAX9, the gradient volume for the elution of WAX9 was only one-half that used to elute cryoprotectin (Fig. 6). Proteins were detected at 220 nm, because cryoprotectin and WAX9 have a low content of aromatic amino acids and therefore detection at 280 nm was not sufficiently sensitive.

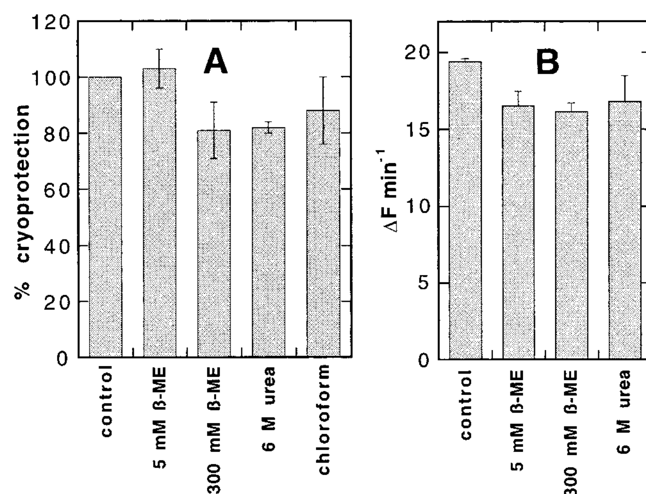

Figure 4. Stability of cryoprotectin (A) and WAX9 (B) in the presence of different protein denaturing substances. After ammonium sulfate precipitation (compare "Materials and Methods"), the proteins were resuspended in 50 mM Tris (pH 7.5; control); 5 mM β-mercaptoethanol (β-ME) in buffer; 300 mM β-ME in buffer; 6 M urea in buffer; or cryoprotectin resuspended in buffer was partitioned against an equal volume of chloroform. After a 15-min incubation on ice, cryoprotectin was transferred into 10 mM Suc, 1 mM $MnCl_2$, and 1 mM $CaCl_2$ by gel filtration chromatography. WAX9 was transferred in the same way into 10 mM Suc. Cryoprotective activity was determined by hematocrit centrifugation for each treatment and the activity recovered in the control samples was set as 100%. Lipid transfer activity is given as the rate of increase in fluorescence emission (ΔF) from the lipid probe NBD-C_{12}-HPC. The data are the means ± SEM from the results of two (cryoprotectin) or three (WAX9) determinations.

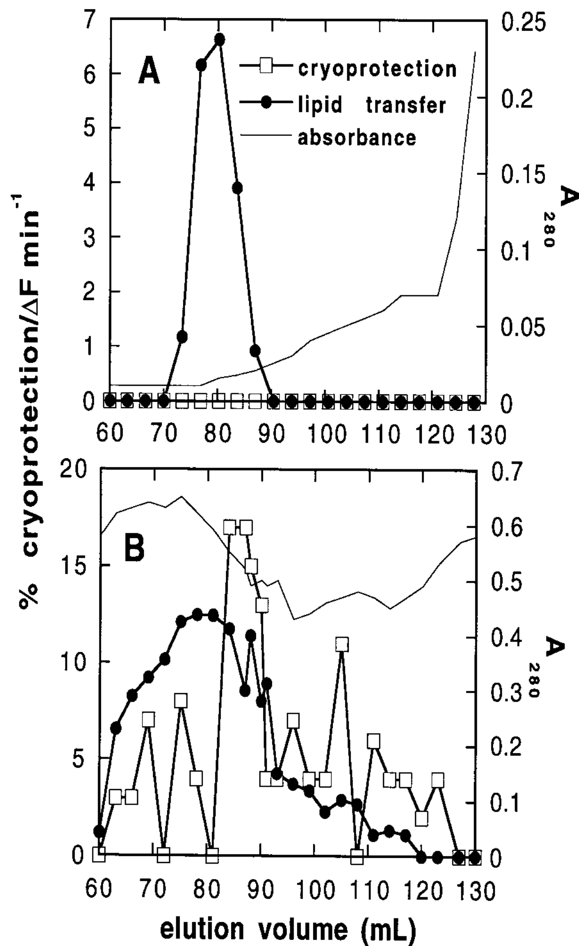

Figure 5. Analysis of crude WAX9 (A) and cryoprotectin (B) preparations from cabbage by gel filtration chromatography. The absorbance of the eluate was continuously monitored at 280 nm and fractions of 3 mL were collected. Lipid transfer activity (ΔF min^{-1}) of the eluted fractions was determined directly. Cryoprotective activity was determined by hematocrit centrifugation after aliquots of the fractions had been transferred into 10 mM Suc, 1 mM $MnCl_2$, and 1 mM $CaCl_2$ by gel filtration through Sephadex G-25 (NAP-5 columns).

Figure 6A shows that WAX9 retained its lipid transfer activity after reverse-phase chromatography. The activity eluted in two distinct peaks that corresponded to absorbance peaks at 220 nm. In contrast, no lipid transfer activity was found after the cryoprotectin sample was fractionated on the same column. Cryoprotectin was not denatured, however, as cryoprotective activity eluted in two clear peaks (Fig. 6B). In this case, no corresponding absorbance peaks could be distinguished. This was probably due to the fact that WAX9 was purified to homogeneity by reverse-phase chromatography, whereas both cryoprotectin peaks still contained other contaminating proteins as judged by SDS-PAGE and silver staining (data not shown). By aminoterminal sequencing, the purified WAX9 protein from cabbage was identified as WAX9 E (Fig. 1). This is different from broccoli where the major cuticular wax protein was identified as WAX9 D (Pyee and Kolattukudy, 1995). It should be emphasized that both cryoprotectin peaks eluted at higher acetonitrile concentrations (28% and 32%) than the major WAX9 peak (22%), indicating that both activities reside in physically different proteins.

Further evidence for functional differences was found in an investigation of the cation dependence of cryoprotective and lipid transfer activities of WAX9 and cryoprotectin. The cyroprotective activity of cryoprotectin was strictly cation dependent. It was inhibited by a pretreatment with EDTA or EGTA (Fig. 7A). Although EDTA complexes most divalent cations, EGTA specifically interacts with Ca^{2+} ions (Schmid and Reilley, 1957). When cryoprotectin was partially inactivated by passage through a Chelex

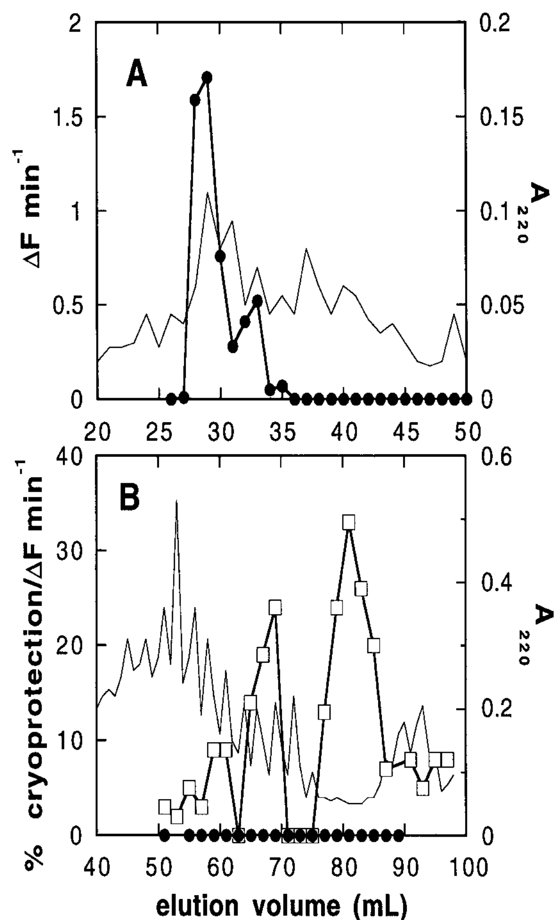

Figure 6. Analysis of WAX9 (A) and cryoprotectin (B) after gel filtration chromatography (compare with Fig. 5) by reverse-phase chromatography. The symbols are the same as in Figure 5. The column was equilibrated in 0.1% (v/v) TFA and proteins were eluted with a linear gradient of acetonitrile (1%–40%) in 0.1% (v/v) TFA. The gradient used to elute cryoprotectin had twice the volume of the gradient used to elute WAX9. The absorbance of the eluate was monitored at 220 nm. The pH in the fractions was readjusted to 7 by the addition of 18 mM Tris (pH 8.6). The fractions (1 mL) were then transferred either into 10 mM Suc, 1 mM $CaCl_2$, and 1 mM $MnCl_2$ (for cryoprotection assays by hematocrit centrifugation), or into 50 mM Tris (pH 7.5) and 200 mM NaCl (for lipid transfer assays) by gel filtration chromatography.

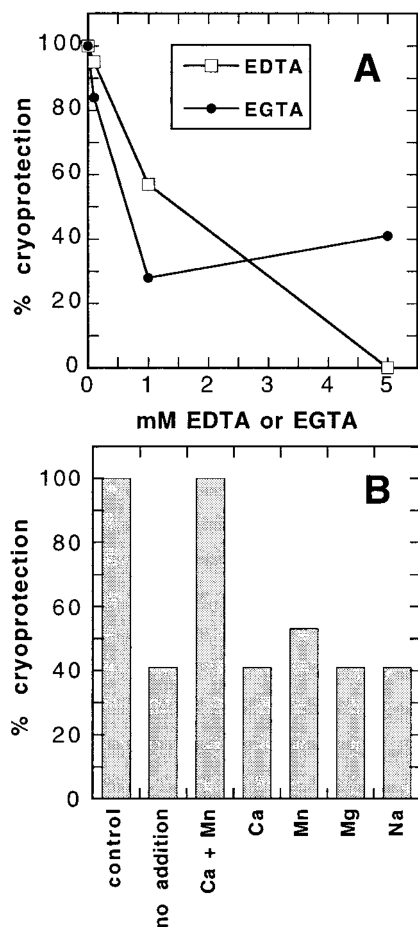

Figure 7. The influence of divalent cations on the cryoprotective activity of cryoprotectin as determined by hematocrit centrifugation. A, Cations were complexed by EDTA or EGTA at the concentrations indicated. The chelators were removed from the samples by gel filtration (NAP-5 columns equilibrated in 10 mM Suc) and the eluted proteins were assayed for cryoprotective activity. Controls containing 5 mM EDTA or EGTA without protein established that no cryotoxic concentrations of these substances were carried over into the samples after gel filtration. The activity of samples that were identically processed without the addition of chelators (0 mM) was set as 100% cryoprotection in this figure. The data are the means from three independent experiments. B, Cryoprotectin was partially inactivated by passage through a Chelex 100 column, which specifically complexes divalent cations. The cryoprotective activity of the sample prior to chromatography was set as 100% in this experiment. To the eluted, partially inactivated cryoprotectin, different cations were added as the chloride salts to a total concentration of 1 mM and the samples assayed for cryoprotective activity.

column, which complexes divalent cations similar to EDTA, the protein could only be reactivated by a combination of $CaCl_2$ and $MnCl_2$. Either salt alone was ineffective, as were $MgCl_2$ or NaCl (Fig. 7B). Neither the presence of EDTA nor Ca^{2+} and Mn^{2+} resulted in any measurable lipid transfer activity in cryoprotectin purified by reverse-phase chromatography. The lipid transfer activity of WAX9, on the other hand, was strongly inhibited in the presence of Ca^{2+} and Mn^{2+}, whereas it was retained in the presence of 5 mM EDTA. Neither addition induced any cryoprotective activity in WAX9 (data not shown).

DISCUSSION

The data presented in Figures 1 through 3 are evidence that cryoprotectin belongs to the structural class of plant LTPs. These proteins are in general characterized by their small molecular mass (between 7 and 10 kD), and eight conserved cystein residues that form four disulfide bridges (Yamada, 1992; Kader, 1996). Six of the eight cystein residues were found in the cryoprotectin sequences at the expected, conserved positions.

The highest degree of sequence identity was found between cryoprotectin and the sequences deduced from the cDNAs of several *wax9* genes (Fig. 1). A WAX9 protein and four different *wax9* cDNA clones were first isolated from broccoli. From sequence comparison it was found that WAX9 is encoded by the *wax9 D* gene in broccoli (Pyee and Kolattukudy, 1995), whereas it is encoded by the *wax9 E* gene in cabbage (Fig. 1). Using PCR primers deduced from the *wax9* cDNA sequences we were able to clone the cabbage homologs of the genes *wax9 A, B, C,* and *D*, previously cloned from broccoli, and in addition a novel member of this gene family, which we termed *wax9 E*. In accordance with previous reports, all *wax9* genes from cabbage coded for proteins with a 25-amino acid leader sequence (Pyee and Kolattukudy, 1995; Fig. 1). WAX9 proteins can be accordingly isolated from the cuticular surface wax of broccoli and cabbage leaves. From this localization and the sequence similarity to other LTPs it was inferred that WAX9 could be involved in the transport of cutin monomers from their epidermal site of synthesis to the leaf surface (Pyee et al., 1994), as had previously been suggested for the LTP-homolog EP2 from carrot (Sterk et al., 1991). Our finding that WAX9 has lipid transfer activity corroborates this hypothesis.

The sequences obtained from cryoprotectin could be aligned to the sequences of WAX9 B, D, and E (Fig. 1) It is not known at present whether all three proteins have cryoprotective activity because we have so far not been able to chromatographically separate these proteins. Our data, however, suggest that the WAX9 proteins comprising cryoprotectin are structurally and functionally different from their leaf surface counterparts. This is primarily indicated by the different in vitro activities and the apparently different localization. WAX9 was isolated by solubilizing the surface wax of cabbage leaves by a brief wash with organic solvents. The purified protein clearly displayed lipid transfer activity between small unilamellar liposomes, but no cryoprotective activity for spinach thylakoids. Cryoprotectin, on the other hand, was isolated from an aqueous leaf extract, and showed no lipid transfer activity, but did show cryoprotective activity (Figs. 5 and 6). It is possible that

cryoprotectin was not able to transfer eggphosphatidylcholine (EPC), but that it is specifically active toward other lipids. This seems unlikely, however, since all plant LTPs investigated so far unspecifically transferred all phospholipids and galactolipids investigated and showed activity with phosphatidylcholine (Kader, 1996). On the other hand, an antimicrobial protein isolated from onion seeds showed sequence homology to LTPs, but no transfer activity for phosphatidylcholine or phosphatidylinositol from liposomes to mitochondria (Cammue et al., 1995). The lack of transfer activity could be attributed to a change in only two amino acids that block the fatty acid binding site in the onion seed LTP (Tassin et al., 1998).

These data indicate that plant LTPs may serve a variety of physiological functions and that some may not require actual lipid transfer activity. For cryoprotectin we would indeed hypothesize that its cryoprotective activity is incompatible with lipid transfer activity. Several researchers have unsuccessfully attempted to purify stable complexes of LTPs with biomembranes or lipid bilayers. The lack of success was interpreted as evidence that LTPs only form unstable, short-lived complexes with the lipids they transfer and that this is actually a prerequisite for transfer activity, as stable binding would preclude rapid transfer. We have found evidence (Sror, Tischendorf, Schmitt, and Hincha, unpublished data) that cryoprotectin binds to thylakoid membranes and that this binding is necessary for cryoprotection. This would indicate that high lipid transfer and cryoprotective activities are mutually exclusive. This is in agreement with the data presented in Figures 5 and 6, which show that although both activities were clearly measurable under our experimental conditions, they were not present in the same proteins. Additional experiments with an LTP partially purified from an aqueous extract of nonacclimated spinach leaves and with two different LTPs from wheat seeds (7 and 9 kD) showed that these proteins also had the expected lipid transfer activity, but no cryoprotective activity (data not shown), indicating that this may be a more general phenomenon.

In addition to these differences in localization and function, there are also several structural features that show that cryoprotectin and WAX9 are similar, but distinctly different proteins. They are very similar in their physico-chemical stability, which may be due to the four intramolecular disulfide bridges in these rather small molecules. Cryoprotectin and WAX9 are not denatured by boiling (data not shown). Heat stability (80°C–100°C) has also been found for LTPs from onion seeds (Cammue et al., 1995), castor bean endosperm (Watanabe and Yamada, 1986), and radish seeds (Terras et al., 1992). Also, cryoprotectin and WAX9 were stable during exposure to 6 M urea, up to 300 mM β-mercaptoethanol, chloroform (Fig. 4), and 0.1% (v/v) trifluoroacetic acid (TFA), and acetonitrile (Fig. 6). The stability of other LTPs toward these chemicals has not been reported.

Differences between the two proteins were apparent in their molecular masses as determined by gel filtration chromatography (Fig. 5). The apparent molecular mass of cryoprotectin as determined on western blots was close to 10 kD (Fig. 2), whereas in silver-stained gels the apparent molecular mass was closer to 7 kD (Sieg et al., 1996). This discrepancy is most likely due to the use of different sets of marker proteins.

The cryoprotective activity of cryoprotectin was strictly dependent on Ca^{2+} and Mn^{2+} and could be completely inhibited with EDTA (Fig. 7). The lipid transfer activity of WAX9, however, was higher in the presence of EDTA than with Ca^{2+} and Mn^{2+}. The elution profiles of the two proteins from a reverse-phase column (Fig. 6) indicate that cryoprotectin is more hydrophobic than WAX9. This greater hydrophobicity may result in a more stable binding of the protein to membrane lipids, as discussed above. This is in accordance with data for cryoprotective lectins, where it was shown that their cryoprotective efficiency was linearly correlated with their relative hydrophobicity (Hincha et al., 1993, 1997c). The lectins participate in hydrophobic interactions with membrane lipids, thereby decreasing lipid fluidity at the membrane surface (Hincha et al., 1997a). This results in reduced solute permeability of the membrane, leading to less solute loading of the membrane vesicles during freezing and therefore to less osmotic rupture during thawing (for review, see Hincha et al., 1996). Whether the cryoprotective effect of cryoprotectin is based on a similar physical mechanism is currently under investigation.

It has been shown that the expression of LTP genes in several plant species is regulated by environmental factors such as cold (Hughes et al., 1992; White et al., 1994; Molina et al., 1996), desiccation (Dunn et al., 1991; Hughes et al., 1992; White et al., 1994; Trevino and O'Connell, 1998), NaCl (Torres-Schumann et al., 1992; Soufleri et al., 1996), pathogens (Hughes and Dunn, 1996; Molina et al., 1996; Nielsen et al., 1996), or heavy metals (Hollenbach et al., 1997). In a similar manner, there is evidence that cryoprotectin is a cold-induced protein (Hincha et al., 1990; Sieg et al., 1996). Our current efforts are directed at clarifying how cryoprotective activity is induced during cold acclimation and whether it is associated with a single protein or with multiple cryoprotectin isoforms.

MATERIALS AND METHODS

Plant Material

Spinach (*Spinacia oleracea* cv Monnopa) was grown under nonacclimating conditions (25°C day/15°C night temperature) in a growth chamber as described in detail recently

(Sieg et al., 1996). Cabbage (*Brassica oleracea* cv Grüfiwi) was grown in the garden for several months. For cold acclimation plants were transferred to pots and held at a constant temperature of 4°C with a 14-h light/10-h dark cycle for 2 weeks.

Purification of Cryoprotectin from the Leaves of Cold-Acclimated Cabbage

We have used two alternative methods for the purification of cryoprotectin. The first steps were identical in both cases and followed the method devised by Sieg et al. (1996). In brief, total soluble protein was extracted from cabbage leaves. The solution was incubated in a boiling water bath for 8 min and was immediately transferred to an ice-water bath. From the cooled solution the precipitated proteins were removed by centrifugation. The supernatant was passed through a column (3 × 60 cm) of Polyamide 6 (Serva, Heidelberg), a matrix consisting of small nylon beads that mainly adsorb phenolic compounds. Cryoprotectin was not bound and the proteins recovered after passage through the column were precipitated by the addition of solid ammonium sulfate to 60% saturation.

To obtain electrophoretically pure cryoprotectin for protein sequencing we followed the method of Sieg et al. (1996) and precipitated the protein with heparin. Heparin (100 mg; Sigma, St. Louis) was added to the resuspended proteins and the mixture was stirred overnight at 4°C. The heparin was removed from the solution by centrifugation (23,000*g* for 15 min) and unbound proteins were removed by washing the pellets twice in 20 mM Na-phosphate (pH 7.3). The elution buffer consisted of 150 mM NaCl, 5 mM β-mercaptoethanol, and 50 mM Tris (pH 7.1). The heparin-cryoprotectin complex was incubated for 30 min at 4°C, followed by centrifugation as above. The eluted proteins were fractionated by reverse-phase chromatography over a C_{18} Vydac column (4.6 × 150 mm; Macherey and Nagel, Düren, Germany) using a LKB (Bromma, Sweden) HPLC system. The proteins were eluted with an acetonitrile gradient from 1% to 60% in 0.1% (v/v) TFA at a flow rate of 0.5 mL min^{-1}.

In an alternate manner, the ammonium sulfate pellets were resuspended in 50 mM Tris (pH 7.5), 200 mM NaCl, and 6 M urea. The proteins were fractionated by gel filtration chromatography on a Superdex 30 (Pharmacia, Uppsala) column (1.6 × 75 cm). The mobile phase was 50 mM Tris (pH 7.5), 200 mM NaCl with a flow rate of 1 mL min^{-1}. The absorbance of the eluate was monitored at 280 nm and fractions of 3 mL were collected. Fractions with cryoprotective activity were pooled and loaded on a reverse-phase chromatography column (POROS10 R2/H, 4.6 × 100 mm; Boehringer Mannheim, Germany). The column was equilibrated in 0.1% (v/v) TFA and proteins were eluted with a linear gradient of acetonitrile (1%–40%) in 0.1% (v/v) TFA at a flow rate of 5 mL min^{-1}. The absorbance of the eluate was monitored at 220 nm. Fractions of 1 mL were collected and the pH in the fractions was readjusted to 7 by the addition Tris (pH 8.6) to a final concentration of 18 mM.

Purification of WAX9

WAX9 was purified from the surface wax of nonacclimated cabbage leaves as described for the same protein from broccoli (Pyee et al., 1994), another variety of *Brassica oleracea*. Leaves (approximately 500 g) were dipped for 20 s in 1.8 L of chloroform:methanol (2:1, v/v). The solvents were removed in a rotary evaporator under reduced pressure at 55°C. The residue was redissolved in 150 mL of chloroform. In a separator funnel, 75 mL of water were added and vigorously mixed with the chloroform. After separation of the phases, the aqueous phase was collected. To precipitate WAX9, ammonium sulfate was added to the aqueous phase (52 g/100 mL; Vergnolle et al., 1992). After stirring for 1 h at 4°C, the precipitated proteins were collected by centrifugation (30 min at 23,000*g*), the pellets were dissolved in gel filtration buffer (50 mM Tris, pH 7.5, 200 mM NaCl), and the proteins were fractionated on a Superdex 30 column as described above for cryoprotectin. Fractions with lipid transfer activity were pooled and applied to a reverse phase column (POROS10 R2/H) as described above for cryoprotectin.

Preparation of Wheat (*Triticum durum*) Seed LTPs

The cDNA (Dieryck et al., 1992) encoding the mature 9-kD LTP from wheat seeds (Désormeaux et al., 1992) was cloned and expressed as a non-fusion protein in *Escherichia coli*. The protein was purified from inclusion bodies (Lullien-Pellerin et al., 1999). The 7-kD LTP from wheat was a generous gift from Dr. D. Marion (Institut National de la Recherche Agronomique, Nantes, France).

Protein Gel Electrophoresis and Western Blotting

Electrophoresis of proteins was performed in polyacrylamide gels under reducing conditions in the presence of SDS as described by Schägger and von Jagow (1987). Polypeptides were stained with silver nitrate (Blum et al., 1987) or were transferred to nitrocellulose membranes by electroblotting (Towbin et al., 1979). Unoccupied binding sites on the membranes were blocked by incubation in 5% (w/v) milk powder, 0.1% (v/v) Tween 20 in 25 mM Tris and 150 mM NaCl (pH 7.5; Johnson et al., 1984). Filters were probed with the anti-LTP antiserum described below. Bound IgG on the filters was visualized with goat anti-rabbit IgG labeled with horseradish peroxidase (Bio-Rad, Hercules, CA) as described by Sieg et al. (1996). The molecular mass of polypeptides was estimated from the position of proteins in a standard mix from Boehringer Mannheim on parallel lanes.

Immunoprecipitation of Cryoprotectin

Antibodies were raised against two synthetic peptides derived from the sequences of different WAX9 proteins (see Fig. 1). The peptides were coupled to keyhole limpet hemocyanin and a mixture of both coupled peptides was injected into two rabbits. Peptide synthesis and antibody preparation were performed by Eurogentec (Seraing, Bel-

gium). For immunoprecipitation, protein A coupled to magnetic beads (Dynal, Oslo) was incubated with the antiserum for 90 min on a shaker. The beads were removed from the solution using a magnetic sample holder (Dynal) and the solution was replaced by a cryoprotectin preparation after ammonium sulfate precipitation in 10 mM Suc, 1 mM $CaCl_2$, 1 mM $MnCl_2$. In some cases the protein solution also contained 8 μg/mL of one of the peptides used for immunization. After another 90-min incubation, the beads were again separated from the solutions in the magnetic sample holder and the supernatants were used for cryoprotection assays and western blotting. Since the amount of preimmune serum that we had available was very limited, we used an anti-Rubisco antiserum as a nonspecific control in the immunoprecipitation experiments. We had ascertained before in western-blot experiments that the preimmune sera from both rabbits showed no interaction with any cabbage polypeptides (data not shown).

Cryoprotection Assays

Thylakoids were isolated from spinach leaves as described (Hincha and Schmitt, 1992a). The membranes were washed three times in 5 mM NaCl. Chlorophyll was determined according to Arnon (1949). Samples (0.4 mL) containing approximately 0.5 mg chlorophyll mL^{-1}, 2.5 mM NaCl, 0.5 mM $MnCl_2$, 0.5 mM $CaCl_2$, 5 mM Suc, and additional protein as indicated in the figures were placed in a freezer at -20°C for 3 h and were rapidly (within 2–3 min) thawed in a water bath at room temperature. During a freeze-thaw cycle under the described conditions, thylakoids rupture and lose their internal contents, e.g. the lumenal protein plastocyanin. They also release their osmotically active solutes and therefore show a smaller volume after thawing. Cryoprotection can be assayed as the retention of vesicle volume or plastocyanin after a freeze-thaw cycle (for review, see Hincha et al., 1992b, 1996).

For the volumetric assay, samples were diluted with an equal volume of 10 mM $MgCl_2$. Aliquots (75 μL) of the thylakoid suspension were filled into glass capillaries and the packed volume was determined by hematocrit centrifugation (Hincha and Schmitt, 1992a). Two measurements were taken from each sample and averaged. Control samples without added protein were held at 0°C (100% protection) or at -20°C (0% protection).

For the plastocyanin release assay (Hincha and Schmitt, 1992a) the membranes were sedimented by centrifugation (15 min at 16,000*g*). The supernatants were subjected to gel electrophoresis and western blotting as described above. Filters were probed with rabbit anti-spinach plastocyanin antiserum (Hincha et al., 1985, 1999) and bound IgG was visualized as described above.

Determination of Cation Dependence of Cryoprotective Activity

A cryoprotectin fraction after polyamide chromatography was transferred into 10 mM Suc, 1 mM $CaCl_2$, and 1 mM $MnCl_2$. To different aliquots of this preparation, EDTA or EGTA was added from concentrated stock solutions. After a 10-min incubation on ice, the chelators were removed by passage of the samples through NAP-5 gel filtration columns equilibrated in 10 mM Suc and the eluted proteins were assayed for cryoprotective activity. As an alternative experimental approach, cation complexation chromatography was used. A cryoprotectin sample after polyamide chromatography was transferred into 10 mM Suc by gel filtration chromatography (NAP-5 columns) and was then passed through a column (1 × 3 cm) of immobilized iminoacetic acid (Chelex 100, Bio-Rad) equilibrated in 10 mM Suc. To aliquots of the eluted proteins, different salts were added from concentrated stock solutions, yielding concentrations of 1 mM $CaCl_2$, $MnCl_2$, $MgCl_2$, or NaCl, or of 0.5 mM each of $CaCl_2$ and $MnCl_2$. These samples were mixed with an equal volume of thylakoids in 5 mM NaCl and assayed for cryoprotective activity.

Measurement of Lipid Transfer Activity

The ability of proteins to transport lipids was assessed as the transfer of fluorescently labeled phosphatidylcholine between liposome membranes (Geldwerth et al., 1991; Moreau et al., 1994). Donor vesicles contained 50 mol% NBD-C_{12}-HPC [(2-(12-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino) dodecanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; Molecular Probes, Leiden, The Netherlands], 40 mol% EPC, and 10 mol% egg phosphatidylglycerol, and acceptor vesicles 90 mol% EPC and 10 mol% egg phosphatidylglycerol (unlabeled lipids were purchased from Sigma). The lipids were mixed in chloroform, dried under a stream of N_2, and stored under vacuum overnight to remove traces of solvent. For donor vesicles, 41 μg of lipid and for acceptor vesicles 4.46 mg of lipid were hydrated each in 500 μL of 10 mM TES [*N*-tris(hydroxymethyl)-2-aminoethanesulfonic acid] buffer (pH 7.4). Small unilamellar liposomes were produced by sonication with a tip sonicator (Branson Sonic Power, Branbury, CT) at 60 W for 6 min in ice water. For transfer measurements, 10 μL each of donor and acceptor vesicles were mixed in a cuvette with 2 mL of 10 mM TES (pH 7.4) and 100 μL of protein solution. Fluorescence was excited at 475 nm and measured at 530 nm in a SFM 25 fluorimeter (Kontron, Neufahrn, Germany). The sample temperature was held constant at 25°C and the solution was continuously stirred with a magnetic stirr bar. At the high concentration in the donor membranes, NBD fluorescence is self-quenched so that fluorescence is minimal at the start of an experiment. When labeled lipid molecules are transferred into acceptor membranes, self-quenching is released and fluorescence emission from NBD is increased. The kinetics of this increase were measured over 1 min. Data were collected by a computer equipped with the appropriate software from Kontron at a rate of 1 s^{-1} and transfer rates were determined by linear regression analysis. Correlation coefficients (r) from linear regression analyses were always better than 0.990. The rates were corrected for the rate of spontaneous transfer in the absence of protein.

Protein Sequencing and Sequence Analysis

Cryoprotectin and WAX9 were N-terminally sequenced. For further internal sequence analysis, cryoprotectin was digested with trypsin in 100 mM *N*-methyl morpholine (pH 8.0) at 37°C overnight. Peptides were purified by reverse-phase chromatography on a Vydac C_{18} TP 218 column eluted with a linear gradient of acetonitrile from 5% to 60% in aqueous 0.1% (v/v) TFA, using a HPLC system (Shimadzu, Columbia, MD). Amino acid sequences were determined by automated Edman degradation on a 473 A gas phase sequencer (Applied Biosystems, Foster City, CA). The obtained sequences were compared with the sequences stored in the EMBL sequence data bank using the HUSAR Swissprot software.

Isolation of mRNA from Cabbage Leaves

Cabbage leaves were ground to a powder with mortar and pestle in liquid nitrogen. From 100 mg of leaf material total RNA was isolated using the RNeasy Plant Mini kit (Qiagen, Valencia, CA). The volume of lysis buffer was doubled. From total RNA, mRNA was isolated using the mRNA isolation kit from Boehringer Mannheim.

Cloning and Sequencing of the *wax9* Genes from Cabbage

cDNA template was generated by reverse transcription of mRNA isolated from cold-acclimated cabbage (First Strand cDNA Synthesis Kit for RT-PCR, Boehringer Mannheim). LTP-specific sequences were amplified with hot-start PCR (0.8 μM primers, 1.5 mM $MgCl_2$, and 1.0 units *Taq* polymerase). The degenerate primers FG11 (RRTGAAGTTGKCATGCTT) and BG297 (AAGGAAYRYTGACTCCACATGC) were chosen using the program Genefisher (http://bibiserv.techfak.uni-bielefeld.de/genefisher/) with the genes for *wax9 A* to *D* as input. After an initial denaturation for 5 min at 94°C, 40 cycles (30 s at 92°C, 45 s at 55°C, and 60 s at 72°C) of amplification were performed. A final extension period of 10 min at 72°C followed. The amplification products were run through an agarose gel and the fragments of the expected size (300–350 bp) were eluted using diethylaminoethyl membranes (NA45, Schleicher & Schuell) and cloned into the T-tailed Eco 321 site of pBluescript (+/-). The resulting clones were differentiated by restriction analysis using the enzymes *Hin*dIII, *Hae*II, and *Xho*I. Some clones with inserts derived from different PCR reactions showed an unusual *Hae*II restriction site. Sequencing revealed a new member of the *wax* gene family, which we named *wax9 E*. The 3′ end of *wax9 E* was obtained using the 3′-/5′-RACE kit (Boehringer Mannheim). PCR amplification of cDNA was done using the oligo dT anchor primer and the gene specific primer RACE-FW (ACTAATAGAAAAATGGCCGGG). The obtained fragments were cloned and checked by restriction analysis using *Hae*II. The 5′ end of *wax9 E* was cloned using the NewRACE procedure (Frohman, 1994) with modifications. cDNAs were sequenced using the Sequenase 2.0 DNA Sequencing Kit and the ΔTaq Cycle Sequencing Kit (United States Biochemical, Cleveland) following the manufacturer's instructions.

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LITERATURE CITED

Arnon DJ (1949) Copper enzymes in isolated chloroplasts: polyphenoloxidase in *Beta vulgaris.* Plant Physiol **24:** 1–15

Artus NN, Uemura M, Steponkus PL, Gilmour SJ, Lin C, Thomashow MF (1996) Constitutive expression of the cold-regulated *Arabidopsis thaliana COR15a* gene affects both chloroplast and protoplast freezing tolerance. Proc Natl Acad Sci USA **93:** 13404–13409

Blum H, Beier H, Gross HJ (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis **8:** 93–99

Cammue BPA, Thevissen K, Hendriks M, Eggermont K, Goderis IJ, Proost P, van Damme J, Osborn RW, Guerbette F, Kader J-C, Broekaert WF (1995) A potent antimicrobial protein from onion seeds showing sequence homology to plant lipid transfer proteins. Plant Physiol **109:** 445–455

Désormeaux A, Blochet J-E, Pézolet M, Marion D (1992) Amino acid sequence of a nonspecific wheat phospholipid transfer protein and its conformation as revealed by infrared and Raman spectroscopy: role of disulfide bridges and phospholipids in the stabilization of the α-helix structure. Biochim Biophys Acta **1121:** 137–152

Dieryck W, Gautier M-F, Lullien V, Joudrier P (1992) Nucleotide sequence of a cDNA encoding a lipid transfer protein from wheat (*Triticum durum* Desf.). Plant Mol Biol **19:** 707–709

Dunn MA, Hughes MA, Zhang L, Pearce RS, Quigley AS, Jack PL (1991) Nucleotide sequence and molecular analysis of the low temperature induced cereal gene, BLT4. Mol Gen Genet **229:** 389–394

Frohman MA (1994) On beyond classic RACE (Rapid Amplification of cDNA Ends). PCR Methods Appli **4:** 40–58

Geldwerth D, de Kermel A, Zachowski A, Guerbette F, Kader J-C, Henry JP, Devaux PF (1991) Use of spin-labeled and fluorescent lipids to study the activity of the phospholipid transfer protein from maize seedlings. Biochim Biophys Acta **1082:** 255–264

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

Hincha DK, Bakaltcheva I, Schmitt JM (1993) Galactose-specific lectins protect isolated thylakoids against freeze-thaw damage. Plant Physiol **103:** 59–65

Hincha DK, Bratt PJ, Williams WP (1997a) A cryoprotective lectin reduces the solute permeability and lipid fluidity of thylakoid membranes. Cryobiology **34:** 193–199

Hincha DK, Heber U, Schmitt JM (1985) Antibodies against individual thylakoid membrane proteins as molecular probes to study chemical and mechanical

freezing damage in vitro. Biochim Biophys Acta **809:** 337–344

Hincha DK, Heber U, Schmitt JM (1990) Proteins from frost-hardy leaves protect thylakoids against mechanical freeze-thaw damage in vitro. Planta **180:** 416–419

Hincha DK, Meins FJr, Schmitt JM (1997b) β-1,3-Glucanase is cryoprotective in vitro and is accumulated in leaves during cold acclimation. Plant Physiol **114:** 1077–1083

Hincha DK, Oliver AE, Crowe JH (1999) Lipid composition determines the effects of arbutin on the stability of membranes. Biophys J **77:** 2024–2034

Hincha DK, Pfüller U, Schmitt JM (1997c) The concentration of cryoprotective lectins in mistletoe (*Viscum album* L.) leaves is correlated with leaf frost hardiness. Planta **203:** 140–144

Hincha DK, Schmitt JM (1992a) Cryoprotective leaf proteins: assay methods and heat stability. J Plant Physiol **140:** 236–240

Hincha DK, Schmitt JM (1992b) Freeze-thaw injury and cryoprotection of thylakoid membranes. *In* GN Somero, CB Osmond, CL Bolis, eds, Water and Life. Springer-Verlag, Berlin, pp 316–337

Hincha DK, Sieg F, Bakaltcheva I, Köth H, Schmitt JM (1996) Freeze-thaw damage to thylakoid membranes: specific protection by sugars and proteins. *In* PL Steponkus, ed, Advances in Low-Temperature Biology. JAI Press, London, pp 141–183

Hollenbach B, Schreiber L, Hartung W, Dietz K-J (1997) Cadmium leads to stimulated expression of the lipid transfer protein genes in barley: implications for the involvement of lipid transfer proteins in wax assembly. Planta **203:** 9–19

Hughes MA, Dunn MA (1996) The molecular biology of plant acclimation to low temperature. J Exp Bot **47:** 291–305

Hughes MA, Dunn MA, Pearce RS, White AJ, Zhang L (1992) An abscisic-acid-responsive, low temperature barley gene has homology with a maize phospholipid transfer protein. Plant Cell Environ **15:** 861–865

Jaglo-Ottosen K, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF (1998) *Arabidopsis CBF1* overexpression induces *COR* genes and enhances freezing tolerance. Science **280:** 104–106

Johnson DA, Gautsch JW, Sportsman JR, Elder JH (1984) Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal Technol **1:** 3–8

Kader J-C (1996) Lipid-transfer proteins in plants. Annu Rev Plant Physiol Plant Mol Biol **47:** 627–654

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nat Biotechnol **17:** 287–291

Krause GH, Grafflage S, Rumich-Bayer S, Somersalo S (1988) Effects of freezing on plant mesophyll cells. Symp Soc Exp Biol **42:** 311–327

Levitt J (1980) Responses of plants to environmental stresses Volume I: Chilling, freezing, and high temperature stresses. Academic Press, Orlando, FL

Lullien-Pellerin V, Devaux C, Ihorai T, Marion D, Pabin V, Joudrier P, Gautier M-F (1999) Production in *Escherichia coli* and site directed mutagenesis of a 9-kDa lipid transfer protein from wheat. Eur J Biochem **260:** 861–868

Molina A, Diaz I, Vasil IK, Carbonero P, Garcia-Olmedo F (1996) Two cold-inducible genes encoding lipid transfer protein LTP4 from barley show differential responses to bacterial pathogens. Mol Gen Genet **252:** 162–168

Moreau F, Davy de Virville J, Hoffelt M, Guerbette F, Kader J-C (1994) Use of a fluorimetric method to assay the binding and transfer of phospholipids by lipid transfer proteins from maize seedlings and *Arabidopsis* leaves. Plant Cell Physiol **35:** 267–274

Nielsen KK, Nielsen JE, Madrid SM, Mikkelsen JD (1996) New antifungal proteins from sugar beet (*Beta vulgaris* L.) showing homology to non-specific lipid transfer proteins. Plant Mol Biol **31:** 539–552

Pyee J, Kolattukudy PE (1995) The gene for the major cuticular wax-associated protein and three homologous genes from broccoli (*Brassica oleracea*) and their expression patterns. Plant J **7:** 49–59

Pyee J, Yu H, Kolattukudy PE (1994) Identification of a lipid transfer protein as the major protein in the surface wax of broccoli (*Brassica oleracea*) leaves. Arch Biochem Biophys **311:** 460–468

Schägger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem **166:** 368–379

Schmid RW, Reilley CN (1957) New complexon for titration of calcium in the presence of magnesium. Anal Chem **29:** 264–268

Sieg F, Schröder W, Schmitt JM, Hincha DK (1996) Purification and characterization of a cryoprotective protein (cryoprotectin) from the leaves of cold-acclimated cabbage. Plant Physiol **111:** 215–221

Soufleri IA, Vergnolle C, Miginiac E, Kader J-C (1996) Germination-specific lipid transfer protein cDNAs in *Brassica napus* L. Planta **199:** 229–237

Steponkus PL (1984) Role of the plasma membrane in freezing injury and cold acclimation. Annu Rev Plant Physiol **35:** 543–584

Steponkus PL, Uemura M, Joseph RA, Gilmour SJ, Thomashow MF (1998) Mode of action of the *COR15a* gene on the freezing tolerance of *Arabidopsis thaliana*. Proc Natl Acad Sci USA **95:** 14570–14575

Sterk P, Booij H, Schellekens GA, van Kammen A, De Vries SC (1991) Cell-specific expression of the carrot EP2 lipid transfer protein gene. Plant Cell **3:** 907–921

Tassin S, Broekaert WF, Marion D, Acland DP, Ptak M, Vovelle F, Sodano P (1998) Solution structure of *Ace*-AMP1, a potent anti-microbial protein extracted from onion seeds: structural analogies with plant nonspecific lipid transfer proteins. Biochemistry **37:** 3623–3637

Terras FRG, Goderis IJ, van Leuven F, Vanderleyden J, Cammue BPA, Broekaert WF (1992) In vitro antifungal activity of a radish (*Raphanus sativus* L.) seed protein homologous to non-specific lipid transfer proteins. Plant Physiol **100:** 1055–1058

Thomashow MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu Rev Plant Physiol Plant Mol Biol **50:** 571–599

Torres-Schumann S, Godoy JA, Pintor-Toro JA (1992) A probable lipid transfer protein gene is induced by NaCl in stems of tomato plants. Plant Mol Biol **18:** 749–757

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA **76:** 4350–4354

Trevino MB, O'Connell MA (1998) Three drought-responsive members of the non-specific lipid-transfer protein gene family in *Lycopersicon pennellii* show different developmental patterns of expression. Plant Physiol **116:** 1461–1468

Uemura M, Steponkus PL (1997) Effect of cold acclimation on membrane lipid composition and freeze-induced membrane destabilization. *In* PH Li, THH Chen, eds, Plant Cold Hardiness: Molecular Biology, Biochemistry, and Physiology. Plenum Press, New York, pp 171–179

Vergnolle C, Arondel V, Jolliot A, Kader J-C (1992) Phospholipid transfer proteins from higher plants. Methods Enzymol **209:** 522–530

Watanabe S, Yamada M (1986) Purification and characterization of a non-specific lipid transfer protein from germinated castor bean endosperms which transfers phospholipids and galactolipids. Biochim Biophys Acta **876:** 116–123

White AJ, Dunn AM, Brown K, Hughes MA (1994) Comparative analysis of genomic sequence and expression of a lipid transfer protein gene family in winter barley. J Exp Bot 1885–1892

Yamada M (1992) Lipid transfer proteins in plants and microorganisms. Plant Cell Physiol **33:** 1–6