

Purification, immunolocalization, cryoprotective, and antifreeze activity of PCA60: A dehydrin from peach (*Prunus persica*)

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Dehydrins are glycine-rich, hydrophilic, heat-stable proteins and are generally induced in response to a wide array of environmental stresses. In previous research (Artlip et al. 1997, *Plant Molecular Biology* 33: 61–70), a full-length dehydrin gene, *ppdh1*, was isolated from peach, and its expression was associated with qualitative and quantitative differences in cold hardiness in sibling genotypes of evergreen and deciduous peach. Similar results were obtained for levels of the corresponding 60 kDa peach dehydrin protein (PCA60). The objective of the present study was to purify the PCA60, test the purified protein for cryoprotective and/or antifreeze activity, and to determine the cellular localization of PCA60 using immunomicroscopy. PCA60 was extracted from winter bark tissues of peach (*Prunus persica* [L.] Batsch) and purified in a two-step process. Separation was based on free-solution

isoelectric focusing followed by size exclusion. Purified PCA60, as well as crude protein extract, preserved the *in vitro* enzymatic activity of lactate dehydrogenase after several freeze-thaw cycles in liquid nitrogen. PCA also exhibited distinct antifreeze activity as evidenced by ice crystal morphology and thermal hysteresis. This is the first time antifreeze activity has been demonstrated for dehydrins. Immunomicroscopy, utilizing an affinity-purified, polyclonal antibody developed against a synthetic peptide of the lysine-rich consensus portion of dehydrins, indicated that PCA60 was freely distributed in the cytoplasm, plastids, and nucleus of bark cells and xylem parenchyma cells. Although the functional role of dehydrins remains speculative, the data support the hypothesis that it plays a role in preventing denaturation of proteins exposed to dehydrative stresses.

Introduction

The dehydrin family of proteins is induced by environmental stresses that result in cellular dehydration (Close 1997). They are glycine-rich proteins, deficient in tryptophan and cysteine, and remain soluble at temperatures approaching 100°C. In addition, dehydrins are characterized by a lysine-rich amino acid sequence present at the carboxy terminus and often repeated throughout the polypeptide. The polypeptide is believed to form an amphipathic helix and might interact with lipids in a manner similar to the interaction of class A amphipathic α -helices of apolipoproteins with lipids, or with exposed hydrophobic patches of partially denatured proteins (Close 1997) in a manner similar to chaperones (Mayhew and Hartl 1996). Thus, dehydrins may

serve a protective function during environmental stress (Dure 1993, Close 1997).

While much has been learned about dehydrins in herbaceous plants, information on dehydrins in woody plants is limited. Seasonal expression of dehydrins has been noted in several species (Wisniewski et al. 1996) including peach (*Prunus persica*) (Arora and Wisniewski 1994) and blueberry (*Vaccinium*, sec. *Cyanococcus*) (Muthalif and Rowland 1994). Chilling and desiccation have also been shown to induce dehydrin gene expression in citrus (*Poncirus trifoliata* and *Citrus grandis*) leaves (Cai et al. 1995). Regarding woody plants, dehydrins have been most extensively characterized in peach, where association of dehydrins with differ-

Abbreviations – AFP: antifreeze protein; BSA: bovine serum albumin; LDH: lactate dehydrogenase; PBS: phosphate buffered saline.

ences in cold hardiness has been documented in different tissues (Arora and Wisniewski 1994, 1996), a dehydrin gene has been cloned and characterized (Artlip, et al. 1997), and its expression in response to drought and ABA have been documented (Artlip and Wisniewski 1997).

In contrast to many herbaceous species that have several dehydrin genes (Close 1997), thus far only one dehydrin gene (*ppdhn1*) and protein (PCA60) have been identified in peach (Arora and Wisniewski 1996, Artlip and Wisniewski 1997, Artlip et al. 1997). PCA60 does not fall into any of the distinct types of dehydrins as described by Close (1997), but appears to represent a hybrid type (Artlip and Wisniewski 1997) and is also one of the larger dehydrins thus far reported (Artlip et al. 1997). It is not known if the hybrid nature of the peach dehydrin is a characteristic of woody plants in general. In an attempt to further characterize PCA60, the present study was undertaken to develop a purification protocol that would allow the determination of whether PCA60 has cryoprotective and/or antifreeze properties *in vitro* and, with the use of dehydrin antibodies, determine the cellular localization of the dehydrin protein. This information is important in order to compare the properties of PCA60 with information on dehydrins reported in herbaceous species and to better understand the possible role of PCA60 in adaptation of peach trees to environmental stress.

Materials and methods

Plant material

All protein preparations used in this study were obtained from bark tissues of current-year-shoots of peach (*Prunus persica* [L.] Batsch) cv. Loring. Bark tissues were scraped from twigs, ground in liquid nitrogen, and stored at -80°C . Similarly, tissues used for immunolocalization studies consisted of current-year-twigs of the same species and cultivar.

Protein purification

Soluble proteins were extracted from bark tissues using a borate buffer (50 mM sodium borate, 50 mM ascorbic acid, 1 mM phenylmethylsulfonyl fluoride [pH 9.0]) at 4°C as previously described (Arora and Wisniewski 1994). Total soluble protein extracts were further fractionated by taking 100 mg of crude extract and separating the proteins based on their isoelectric point using a Bio-Rad Rotofor Cell (Bio-Rad, Hercules, CA, USA) under denaturing conditions as described by Neven et al. (1992). Proteins were focused for 4.5 h at 15 W with 4°C coolant. After focusing, samples (20 fractions) were collected. A 5.0- μl aliquot from each fraction was separated by SDS-PAGE and stained with Coomassie brilliant blue G-250 using the procedure of Neuhoff et al. (1988). As in a previous study (Arora and Wisniewski 1994), fractions in the pH range 7.2–7.6 were highly enriched in PCA60. These fractions were pooled and prepared for further purification. This was accomplished by separating the PCA60-enriched fractions by molecular mass using a Bio-Rad Model 491 Prep Cell (Bio-Rad, Hercules, CA, USA), containing a 9% acrylamide gel, according to the manufac-

turer's directions. The flow rate was adjusted to 1.0 ml min^{-1} and 3.0-ml fractions were collected. A total of 10 μl from each fraction was separated using SDS-PAGE with a 12.5% gel and stained with Coomassie blue G-250. Fractions containing only the PCA60 were pooled, dialyzed against either 10 mM Tris-HCl (pH 7.5) or distilled water, and lyophilized to reduce the total volume. Protein estimations were conducted by a modified Bradford assay (Ramagl and Rodriguez 1985) and with dotMetric™ (Geno Technology, St. Louis, MO, USA) protein assay kit. Both methods gave similar results. The latter kit, whose quantitation is not dependent on the amino acid composition of the protein, was used to verify the results of the Bradford assay, which can be biased to proteins having basic and aromatic amino acid residues, thus resulting in protein-to-protein variation. The purified PCA60 obtained was used to conduct both the cryoprotective and antifreeze assays.

Cryoprotection assay

The protocol for assessing cryoprotective activity of crude protein extract and purified PCA60 *in vitro* was based, with slight modification, on the method described by Carpenter and Crowe (1988) and used by Lin and Thomashow (1992). Lactate dehydrogenase (Sigma, St. Louis, MO, USA) was dialyzed against 10 mM potassium phosphate (pH 7.5) in order to remove any stabilizers used by the manufacturer and then diluted to 0.3 mg ml^{-1} and used as a stock solution. Samples of either PCA60 or bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA) were diluted to varying concentrations and mixed with 5 μl of lactate dehydrogenase (LDH) from the stock solution. The resulting solutions were frozen in liquid nitrogen for 30 s and then thawed at ambient temperature for 5 min. The freeze-thaw process was conducted nine times which ensured almost no enzyme activity in the control samples (LDH without any added protein). Data are reported as percent activity compared with an unfrozen control. Three samples were used for each concentration of protein tested and the experiment was repeated several times.

Immunomicroscopy

Procedures used to determine the cellular localization of dehydrin in peach tissues were similar to those described by Egerton-Warburton et al. (1997). Current-year-shoots of peach (cv. Loring) were collected in mid-winter (December and January). Transverse twig sections were cut to ≈ 2 –3 mm in thickness and fixed in 5% (v:v) glutaraldehyde in 25 mM sodium phosphate buffer (pH 6.8) for 4 h as previously described (Wisniewski and Davis, 1995). Samples were then dehydrated in a graded ethanol series and embedded in epoxy resin. Samples were cut on an ultramicrotome for light microscopy and mounted on glass slides. Subsequently, specimens were trimmed down to an area of interest, sectioned for transmission electron microscopy and mounted on 200 mesh nickel grids.

For light microscopy, 25- μl droplets of each of the described solutions were placed directly over an area of the glass slide containing mounted specimens. Sections were

preblocked for 1 h using 0.01% (v:v) SDS in phosphate buffered saline (PBS). Samples were then rinsed with PBS and the blocking solution was replaced with affinity-purified antidehydrin IgG diluted 1:50 (v:v) (Egerton-Warburton et al. 1997). The dehydrin antibody was directed against a synthetic peptide comprised of a 15 amino acid consensus sequence for dehydrins (Close et al. 1993). The consensus sequence is repeated nine times in the sequence of PCA60. Sections were incubated for 2 h, rinsed three times in PBS and incubated for 2 h in goat-antirabbit IgG conjugated to 10 nm gold particles diluted 1:100 (v:v). Pre-immune serum was substituted for the dehydrin antibody for determination of non-specific labeling. Alternatively, sections were labeled with a solution of dehydrin antibody that had been mixed with the peptide used to generate the antibodies as described in Egerton-Warburton et al. (1997). Sections were then silver-enhanced for 3–5 min according to the manufacturer's instructions (BB International, Cardiff, UK) and sealed under a coverslip using Permount. Sections were photographed using a Zeiss Axiophot compound microscope.

Labeling of ultra-thin sections for transmission electron microscopy was carried out by floating grids on 10- μ l droplets placed in sterile petri dishes. Controls included samples treated only with secondary antibody and samples treated with pre-immune serum instead of primary antibody. Sections for electron microscopy were also silver-enhanced in order to visualize the pattern of labeling at low magnification. Labeled sections were then viewed either unstained or after a brief (10 min) staining with uranyl acetate using a Hitachi H-600 transmission electron microscope at 50–75 kV.

Assay of antifreeze activity

Antifreeze activity was determined qualitatively by observing the growth of ice crystals in solution using a nanoliter osmometer (Clifton Technical Physics, Hartford, NY, USA) and a phase-contrast photomicroscope (Olympus BHT) as described in detail by Hon et al. (1994). In this system, an ice crystal grown in water forms a flat disc as the crystal grows normal to the *c*-axis. When grown in the presence of antifreeze proteins that bind to the surface of the ice, the crystal becomes hexagonal in shape as crystal growth is inhibited along the *a*-axes. At high concentrations (micromolar to millimolar) of antifreeze proteins, the *c*-axis becomes the preferred direction of growth and the crystal forms a hexagonal bipyramid (De Vries 1986). In the experiments reported here, PCA60 was dialyzed against distilled water, lyophilized, resuspended in a minimal volume of distilled water and assayed for antifreeze activity.

Antifreeze activity was also assayed quantitatively by determining the level of thermal hysteresis, or noncolligative freezing point depression. In this case, a single, stable ice crystal was produced in a solution containing 1.7 μ g PCA60 μ l⁻¹ 10 mM Tris. The temperature of the solution was lowered until the ice crystal started to grow, which was taken as the freezing temperature. The solution was then warmed until the faces of the ice crystal became round: this was taken as the melting temperature. The assay was re-

peated on three individual crystals. Thermal hysteresis was calculated as the difference between the freezing and melting temperatures of the ice crystals.

In order to ascertain that the antifreeze activity was a result of a protein, PCA60 (0.4 μ g protein μ l⁻¹ distilled water) was denatured by heating the solution to 96°C for 15 min and repeating the antifreeze assay. PCA60 was also degraded by adding 3 μ g proteinase K (Sigma, St. Louis, MO, USA) μ l⁻¹ of solution containing 0.4 μ g PCA60 μ l⁻¹ distilled water and the solution was incubated at 25°C for 5 min before repeating the antifreeze assay.

Results

Protein purification

As demonstrated in a previous report (Arora and Wisniewski, 1994), fractionization of the crude peach bark protein extract by free-solution isoelectric focusing (IEF) in a Rotofor apparatus resulted in some fractions that were highly enriched in PCA60 (Fig. 1A). Specifically, out of a total of 20 fractions (pH 3.1–9.3), fractions 15 and 16 had an isoelectric point of 7.3 and were composed of >80% PCA60, based on staining intensity in the gel. The identity of the PCA60 was confirmed by immunoblot analysis (data not shown).

Further purification was carried out by pooling PCA60-enriched fractions and running them through a Model 491 Prep Cell (Bio-Rad, Hercules, CA, USA) utilizing a 9%

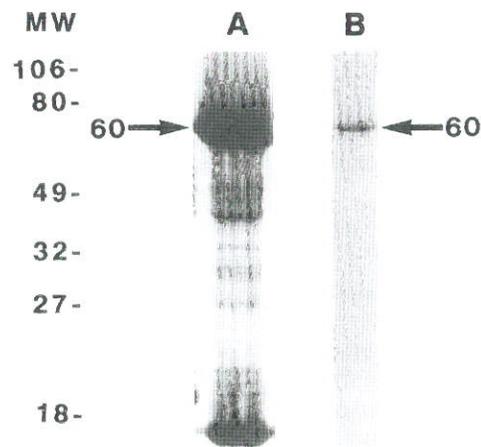


Fig. 1. Purification of PCA60, a peach bark dehydrin. A. Fraction 16 of total soluble bark protein separated by free-solution isoelectric focusing using a Rotofor (Bio-Rad, Hercules, CA, USA). Approximately 100 mg of crude protein extract was loaded in the Rotofor Cell under denaturing conditions. The resulting 20 fractions were further separated using SDS-PAGE (12.5% gel) where each lane represented a different Rotofor fraction. Identification of PCA60 was confirmed by immunoblot analysis (data not shown). The fraction is highly enriched in PCA60. B. Further purification of PCA60. Fractions obtained from the Rotofor Cell containing PCA60 were pooled and further separated based on molecular mass using a free-solution Model 491 Prep-Cell (Bio-Rad, Hercules, CA, USA). Fractions from the Prep-Cell containing only PCA60 were identified by SDS-PAGE (12.5% gel) and confirmed by immunoblot analysis. The 60-kDa protein that is present at near homogeneity is PCA60. These fractions were then pooled, dialyzed against 10 mM Tris-HCl (pH 7.5) and concentrated prior to storage at -20°C.

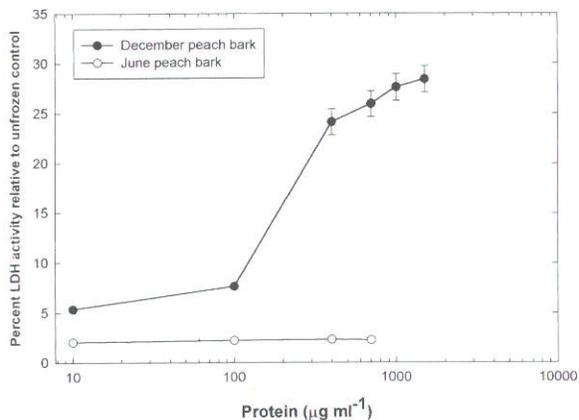


Fig. 2. Cryoprotection of lactate dehydrogenase (LDH) by the addition of different concentrations (log scale) of soluble extracts of peach bark proteins. The curve shows the percentage LDH activity remaining after freeze-thaw deactivation relative to an unfrozen control. (Mean \pm SE, $n = 3$).

acrylamide gel. This device provided further separation based on molecular mass. Proteins in the resulting fractions were identified using SDS-PAGE. Fractions containing just the PCA60 (Fig. 1B) were pooled, dialyzed against 10 mM Tris-HCl (pH 7.5) buffer, and lyophilized for use in the cryoprotective, and antifreeze assays. In general, the purification procedure was simple and resulted in obtaining PCA60 that retained activity. Starting with ≈ 100 mg of crude protein extract, we were able to obtain ≈ 500 – 700 μg of PCA60.

Cryoprotection assay

The freeze-thaw protocol used in this study resulted in a $> 90\%$ loss in LDH activity (Figs. 2 and 3), which provided a basis for testing cryoprotective activity of peach bark proteins. When added to the LDH solutions prior to freeze-thaw protocol, extracts of total soluble protein obtained from peach bark collected in June had no crypro-

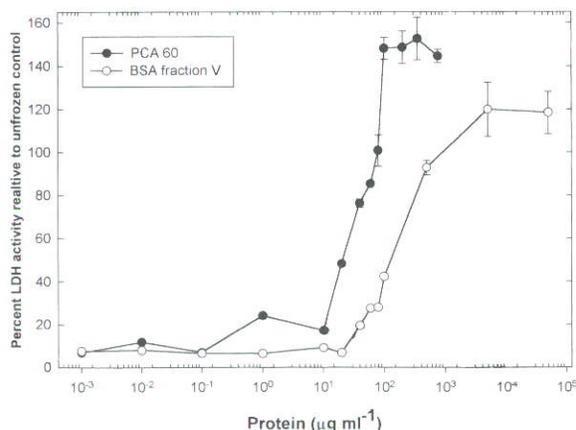


Fig. 3. Cryoprotection of lactate dehydrogenase (LDH) by PCA60. The curve shows the percentage LDH activity remaining after freeze-thaw deactivation in the presence of different concentrations (log scale) of PCA60 or bovine serum albumin (BSA) relative to an unfrozen control. (Mean \pm SE, $n = 3$).

protective activity (Fig. 2). In contrast, 29% of the LDH activity present in unfrozen controls was maintained by adding ≈ 1 mg of the crude total soluble protein from peach bark collected in December. These results indicate that cryoprotective substances accumulate in peach bark tissues during cold acclimation in the fall.

A significant increase in cryoprotective activity was observed when purified PCA60 was used instead of crude extract (Fig. 3). In comparison with BSA, PCA60 was about ten times more active on a mass basis. Whereas, ≈ 20 $\mu\text{g ml}^{-1}$ of PCA60 maintained 50% of LDH activity, 100 $\mu\text{g ml}^{-1}$ of BSA was needed to maintain the same level of activity. Similarly, 100 $\mu\text{g ml}^{-1}$ of PCA60 prevented any loss of LDH activity, whereas 1000 $\mu\text{g ml}^{-1}$ of BSA was required to obtain the same results. Interestingly, with both PCA60 and BSA activity levels $> 100\%$ (150 and 120%, respectively) were obtained compared with the activity of unfrozen controls. In comparison, the addition of as much as 60 mg ml^{-1} of sucrose, a commonly used cryoprotectant, resulted in only 42% LDH activity following freeze-thaw inactivation (data not shown).

Immunomicroscopy

The localization of PCA60 in peach tissues was evaluated with light and electron microscopy using immunogold-labeled sections that had been silver-enhanced (Figs. 4 and 5). Controls consisting of either sections exposed to pre-immune serum rather than dehydrin antibody, or exposed to a mixture of dehydrin antibody and the peptide used to develop the dehydrin antibody, did not reveal any non-specific labeling (Fig. 4A,B, respectively). Sections labeled with the dehydrin antibody indicated a general distribution of PCA60 in cells of all tissues of current-year-shoots collected in January (Fig. 4C,D) when PCA60 levels are known to be high. This included epidermal, cortical, phloem, and xylem tissues. In bark tissues, heavier labeling was observed in the 4–5 cell layers subtending the epidermis (Fig. 4C). Higher magnifications indicated that label was present throughout the cytosol and also in nuclei, but absent from vacuoles (Fig. 4D). Similar results were observed in ray parenchyma cells of xylem tissues (Fig. 4E,F).

Ultrastructural observations further confirmed the general distribution of PCA60 (Fig. 5A,B). Label was generally distributed throughout the cytosol, within the nucleus, and also within chloroplasts and to a lesser extent other organelles (Fig. 5A–D). Within the nucleus, label was apparent in both heterochromatin and euchromatin, although mostly in the former (Fig. 5E,F). The nucleolus appeared to have the heaviest amount of label in all cell types whereas vacuoles were virtually devoid of any label (Fig. 5).

Antifreeze activity

As shown in Fig. 6A,B, PCA60 exhibited the ability to modify the normal growth of an ice crystal. The formation of ice crystals shaped as hexagonal bipyramids in solutions

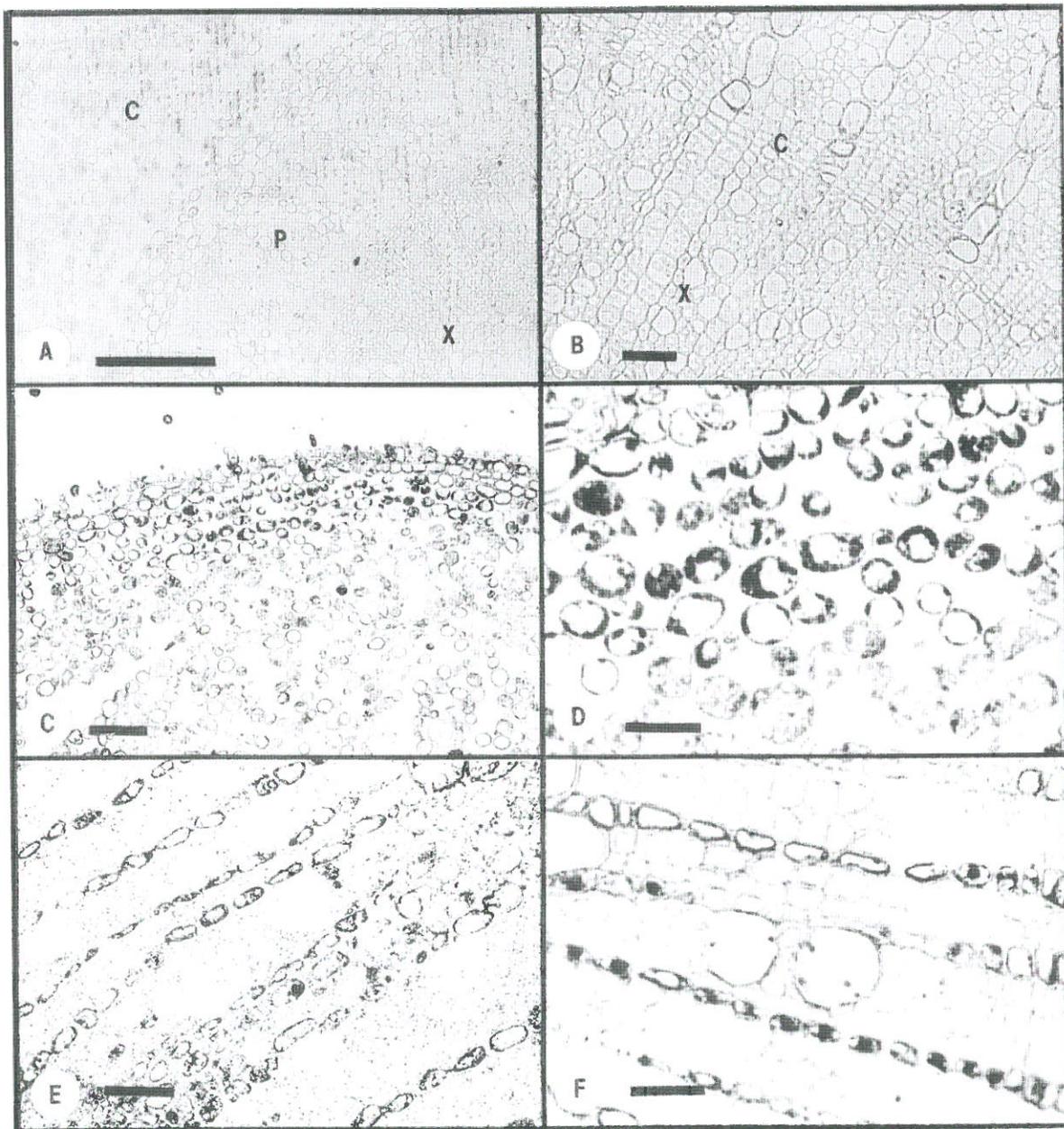


Fig. 4. Silver-enhanced, immunogold localization of PCA60 (peach dehydrin) in shoot tissues of peach collected in January. Labeling studies utilized a dehydrin antibody directed against a synthetic 20-mer peptide representing the carboxy-terminus consensus sequence of dehydrins. A. Tissue section probed with pre-immune serum. B. Tissue section probed with a mixture of dehydrin antibody and the synthetic peptide used to generate the antibody. C. Positive labeling of dehydrin in cortical tissues of peach. D. General distribution of dehydrin in cortical cells. Note absence of label in vacuoles. E. Positive labeling of dehydrin in xylem ray parenchyma cells of peach. F. General distribution of dehydrin in xylem ray parenchyma cells. Note absence of label from vacuoles. C, cortical tissue; P, phloem tissue; X, xylem tissue. Bar = 50 μm . A: 10 μm , B, D, E, F; 20 μm , C.

of PCA60 indicated a fairly high level of antifreeze activity. PCA60 also exhibited $0.06 \pm 0.03^\circ\text{C}$ of thermal hysteresis in a dilute solution containing $1.7 \mu\text{g PCA60 } \mu\text{l}^{-1}$ distilled water. When PCA60 was treated with heat or with proteinase K, the antifreeze activity disappeared (Fig. 6C,D). These results show that PCA60 fulfills the definition of an antifreeze protein in that it has the ability to modify the normal growth of ice and it exhibits thermal hysteresis. In addition, the above results show that a relatively intact protein is required for these activities.

Discussion

In order to further characterize the dehydrin found in winter bark and xylem tissues of peach, a simple procedure was developed for purifying the protein. Using a Rotofor (Bio-Rad, Hercules, CA, USA) for free-solution isoelectric focusing followed by further separation based on molecular mass, utilizing a Model 491 Prep Cell (Bio-Rad, Hercules, CA, USA), 300–500 μg of dehydrin protein was recovered from 100 mg of crude protein extract, representing a 0.3–0.5%

recovery rate. We estimate that the final product represents >98% PCA60 based on the protein profiles visible on heavily loaded SDS-PAGE gels (Fig. 1B) and immunoblots. The method described is simpler than the method described by Ceccardi et al. (1994) for purification of maize dehydrin, and does not involve heating the sample at elevated temperatures (~75°C). The authors method, however, may not result in purification to complete homogeneity and does

expose the native protein to denaturing conditions during the purification. Despite exposure to denaturing conditions (SDS), PCA60 retained activity as evidenced in the LDH freeze-thaw inactivation and antifreeze assays.

COR15a, a cold-regulated, heat-stable protein in *Arabidopsis* that is targeted to the chloroplast, has been shown to have potent cryoprotective activity *in vitro* using the LDH assay (Lin and Thomashow 1992). The results from

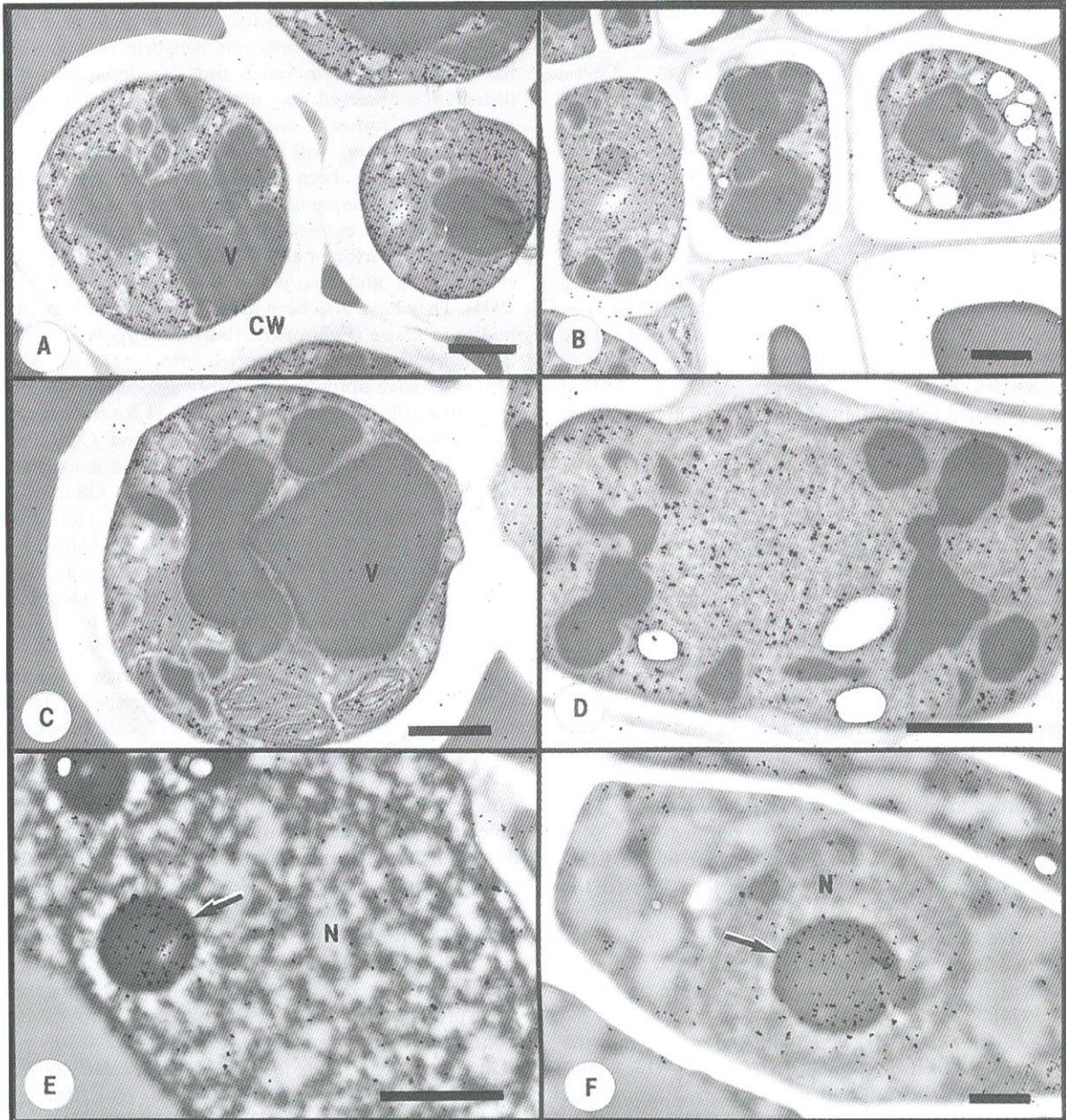


Fig. 5. Ultrastructural localization (using silver-enhanced, immunogold microscopy) PCA60 in cortical and xylem parenchyma cells of current-year-shoots of peach collected in January. The primary antibody was developed against a 20-mer synthetic peptide of the carboxy-terminus consensus sequence of dehydrins. A. Cortical cell showing general distribution of dehydrin in the cytosol. Note absence of label in vacuoles. B. Xylem parenchyma cell showing general distribution of dehydrin in the cytosol, and within nuclei. Note absence of label in vacuoles. Apparent labeling in the cell wall is precipitate from the silver enhancing process and does not represent labeling of dehydrin. C. Cortical cell showing labeling of dehydrin in cytosol and within plastids. D. Xylem parenchyma cell showing labeling of dehydrin in the cytosol and within the nucleus. E. Cortical cell showing labeling of dehydrin within the nucleus and nucleolus. F. Xylem parenchyma cell showing labeling of dehydrin within the nucleus and nucleolus. CW, cell wall; V, vacuole; N, nucleus. Bar = 2.5 μ m, A–D; 2.0 μ m, E; 1.0 μ m, F.

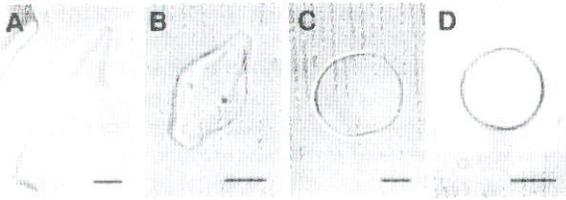


Fig. 6. Modification of ice crystal growth by PCA60. A, B. Representative examples of single ice crystals grown in solutions containing $0.4 \mu\text{g PCA60 } \mu\text{l}^{-1}$ distilled water using a nanoliter osmometer. The c -axis of each ice crystal is parallel to the plane of the page and extends diagonally from the lower left corner to the upper right corner. These crystals are hexagonal in cross-section. C. Ice crystal formed after a solution containing $0.4 \mu\text{g PCA60 } \mu\text{l}^{-1}$ distilled water was heated to 96°C for 15 min. D. Ice crystal formed after $3 \mu\text{g proteinase K } \mu\text{l}^{-1}$ distilled water was added to a solution containing $0.4 \mu\text{g PCA60 } \mu\text{l}^{-1}$ distilled water and the solution was incubated at 25°C for 5 min. In C and D, the c -axes of the ice crystals are perpendicular to the plane of the page. Magnification bar represents $10 \mu\text{m}$.

the present study indicate that PCA60, a peach dehydrin, also has cryoprotective activity but not as strong (on a concentration basis), as that reported for COR15a. For example, 50% of LDH activity is maintained when $0.1 \mu\text{g ml}^{-1}$ of COR15a is present in the enzyme solution prior to the freeze-thaw protocol. In contrast, $\approx 20 \mu\text{g ml}^{-1}$ of PCA60 is required to maintain 50% activity. This represents an activity 5-fold greater than BSA (Fig. 3), whereas COR15a exhibits cryoprotective activity 100-fold higher than BSA (Lin and Thomashow 1992). It should be noted that the data reported for COR15a represented protein obtained from an *in vitro* expression system producing the complete protein coded for the COR15a gene. Subsequent work using the mature form of the protein in which a portion of the protein is cleaved indicated that the cryoprotective activity of COR15a was similar to that of BSA (Artus et al. 1996; M. Thomashow, personal communication). Results presented here are similar, however, with those reported for both COR85 (Kazuuoka and Oeda 1994) and Wcs120 (Houde et al. 1995), dehydrins from spinach (*Spinacia oleracea*) and wheat (*Triticum aestivum*), respectively. In order to maintain 50% LDH activity following a freeze-thaw cycle, either $10 \mu\text{g ml}^{-1}$ of purified Wcs120 or $15 \mu\text{g ml}^{-1}$ of purified COR85 was needed in the LDH reaction mixture. Indirect evidence to further support the cryoprotective activity of PCA60 is the observation that no cryoprotective activity was observed in crude protein extracts from summer months (Fig. 2) when PCA60 is known to be absent from bark tissues of peach (Arora and Wisniewski 1994).

LDH activities $>100\%$ were obtained with both the putative cryoprotective proteins COR15a (Lin and Thomashow 1992), PCA60 and with BSA, indicating that these proteins have a stabilizing effect on LDH. In addition, sucrose, a commonly used cryoprotectant, had little cryoprotective activity compared to the proteins tested requiring $\approx 60 \text{ mg ml}^{-1}$ (175 mM) to maintain 50% LDH activity. Similar results were obtained by Lin and Thomashow (1992) and Houde et al. (1995).

Immunolocalization studies indicated that the affinity-purified antidehydrin IgG specifically recognized PCA60.

This conclusion is supported by the fact that there was no labeling observed in the control samples incubated with pre-immune serum or a mixture containing dehydrin antibody and the synthetic protein used to generate the antibody (Fig. 4A,B). In both types of controls labeling was negligible. A detailed morphometric analysis of the ability of this antibody to recognize maize dehydrin has recently been reported (Egerton-Warburton et al. 1997). The cytosolic and nuclear distribution of PCA60 in bark and xylem tissues of current-year-shoots of peach is, collectively, in agreement with the distribution reported in herbaceous plants (Close 1996). As with maize, only one dehydrin protein and gene has been identified in peach thus far, indicating that the distribution observed may not represent a family of related proteins but rather a single, specific dehydrin. Additional research, however, will be needed to verify this premise. Dehydrins have been associated with the cytosol in *Craterostigma plantagineum* (Schneider et al. 1993) and with the nucleus and cytosol in *Zea mays* (Asgar et al. 1994, Egerton-Warburton et al. 1997), wheat crown tissue (Houde et al. 1995), and *Lycopersicon esculentum* (Godoy et al. 1994). They have also been associated with protein and lipid bodies in maize (Egerton-Warburton et al. 1997) indicating that dehydrins may be imported into organelles. Although dehydrins have not been previously associated with plastids, two other cold-induced proteins, Dsp34 from *Craterostigma plantagineum* (Schneider et al. 1993) and COR15a from *Arabidopsis*, (Artus et al. 1996) have been associated with the thylakoid membranes of chloroplasts. COR15a has also been shown to enhance *in vivo* freezing tolerance of chloroplasts and protoplasts (Artus et al. 1996). Danyluk et al. (1998) also indicated that a cold-induced, acidic dehydrin (WCOR410) may localize to the plasma membrane in wheat. While a detailed discussion of the putative functional role of dehydrins is not within the scope of the present report, the available data support the view proposed by Egerton-Warburton et al. (1997) that the specific biochemical activity of dehydrins (based on its localization) is likely to be diverse (Campbell and Close 1997).

A novel and original finding of this research is the antifreeze activity observed for PCA60 (Fig. 6). This is the first time that antifreeze activity has been demonstrated for a dehydrin. To date, plant antifreeze proteins have only been purified from winter rye *Secale cereale* (Hon et al. 1994) and from bittersweet nightshade *Solanum dulcamara* (Duman 1994). The proteins these plants are very different; winter rye antifreeze proteins (AFP) are polypeptides ranging in size from 16 to 35 kDa with similarity to pathogenesis-related proteins (Hon et al. 1995), whereas the AFP isolated from the woody tissues of bittersweet nightshade is a 67-kDa glycoprotein (Duman 1994). PCA60 clearly exhibits an affinity for the surface of ice (Fig. 6) and depresses the freezing temperature noncolligatively, so it is also an AFP. This is the first known example of a dehydrin with the ability to modify the growth of ice. In support of the observation of antifreeze activity by PCA60 is the prediction that the secondary structure of dehydrins should be amphipathic α -helical, which is also a characteristic of Type I, AFP from fish (Hew and Yang 1992). It is not unusual to have discovered different types of AFPs in plants, as four

distinct types of AFPs have been characterized in polar fish (Davies and Hew 1990). Each type of AFP is thought to have evolved independently as different organisms adapted to exposure to freezing temperatures.

With respect to its antifreeze activity, the unusual characteristic of PCA60 is that it is located within cells in both the cytoplasm and nucleoplasm. In fish, AFPs are synthesized in the liver and secreted into the blood, where they are carried throughout the organism by the circulatory system (Gong et al. 1996). In insects, AFPs also circulate within the hemolymph (Duman et al. 1993). In both cases, it is presumed that the AFPs inhibit the growth of extracellular ice crystals. This is also the case in winter rye leaves, as AFPs are found exclusively in the apoplast where ice forms during freezing (Pihakaski-Maunsbach et al. 1996). Only the winter rye AFP that is similar to β -1,3-endoglucanase has been localized to the subcellular level using immunogold, and it is localized in the cell walls, middle lamellae and pectinaceous cell wall junctions of leaves (Pihakaski-Maunsbach et al. 1996). There is no evidence for the accumulation of rye AFPs within the cells as they are not detectable in protoplasts and, when localized using immunogold, they are only visible in the secretory pathway within the cell. Thus, the presence of an intracellular AFP in rye seems unlikely. However, an intracellular AFP has recently been cloned from the skin of winter flounder, where it is believed to function as a barrier to the penetration of ice through the skin of fish swimming in ice-laden seawater (Valerio et al. 1992, Gong et al. 1996). The biological significance of the antifreeze activity of PCA60 is uncertain. It may simply be fortuitous, brought about as a consequence of its structure. As a result of its hydrophilicity and concomitant ability to bind water, the peach dehydrin may bind to ice surfaces and inhibit the ability of water molecules to join ice lattices, as has been generally proposed for AFPs (Caple et al. 1996). AFPs from fish and plants have been shown to effectively inhibit ice nucleators (Parody-Morreale et al. 1988, Zamecnik and Janacek 1992). Therefore, it is also possible that the accumulation of dehydrins within xylem ray parenchyma may inhibit intracellular ice nucleation and promote supercooling in these cells.

Dehydrins are usually glycine-rich, hydrophilic, heat-stable proteins that are induced by a wide array of environmental stresses (Close 1996). PCA60 is a dehydrin protein whose expression is seasonally regulated in bark and xylem tissues of peach and whose presence is associated with increased levels of cold hardiness within and between genotypes (Arora and Wisniewski 1994, Artlip et al. 1997). Similar to other dehydrins, it is glycine rich, hydrophilic, and heat-stable. The gene encoding PCA60 encodes 472 amino acids with a predicted mass of 50020 Da, contains nine lysine-rich repeats characteristic of dehydrins, and two DEYGNP motifs at the amino acid terminus (Artlip et al. 1997). FTIR analysis supports the prediction that the protein is primarily helical with no evidence for β sheets (C. Stushnoff, personal communication). The present study provides a simple procedure for purifying PCA60 to near homogeneity and has provided evidence that PCA60 has both cryoprotective and antifreeze activity. The induction of dehydrins by dehydrative stress, their abundance, cellular

distribution, predicted structural properties, and their cryoprotective activity, all support the idea that dehydrins moderate the detrimental effects of dehydration.

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References

- Arora R, Wisniewski M (1994) Cold acclimation in genetically related (sibling) deciduous and evergreen peach (*Prunus persica* [L.] Batsch.). II. A 60-kilodalton bark protein in cold acclimated tissues of peach is heat stable and related to the dehydrin family of proteins. *Plant Physiol* 105: 95–101
- Arora R, Wisniewski M (1996) Accumulation of a 60 kD dehydrin in peach xylem tissues and its relationship to cold acclimation. *Hort Sci* 31: 923–925
- Artlip T, Wisniewski M (1997) Tissue-specific expression of a dehydrin gene in one-year-old 'Rio Oso Gem' peach trees. *J Am Soc Hort Sci* 122: 784–787
- Artlip T, Callahan A, Bassett C, Wisniewski M (1997) Seasonal expression of a dehydrin gene in sibling deciduous and evergreen genotypes of peach. *Plant Mol Biol* 33: 61–70
- Artus NN, Uemura M, Steponkus PL, Gilmour S, 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
- Asghar R, Fenton RD, DeMason DA, Close TJ (1994) Nuclear and cytoplasmic localization of maize embryo and aleurone dehydrin. *Protoplasma* 177: 87–94
- Cai Q, Moore GA, Guy CL (1995) An unusual group 2 LEA gene family in citrus responsive to low temperature. *Plant Mol Biol* 29: 11–23
- Campbell S, Close TJ (1997) Dehydrins: Genes, proteins, and associations with phenotypic traits. *New Phytol* 137: 61–74
- Caple G, Kerr WL, Burcham TS, Osuga DT, Yeh Y, Feeney RE (1996) Superadditive effects in mixture of fish antifreeze glycoproteins and polyalcohols or surfactants. *J Colloid Interf Sci* 11: 299–304
- Carpenter JF, Crowe JH (1988) The mechanism of cryoprotection of proteins by solutes. *Cryobiology* 25: 244–255
- Ceccardi TL, Meyer NC, Close TJ (1994) Purification of a maize dehydrin. *Protein Exp Purif* 5: 266–269
- Close TJ (1996) Dehydrins: Emergence of a biochemical role of a family of plant dehydration proteins. *Physiol Plant* 97: 795–803
- Close TJ (1997) Dehydrins: A commonality in the response of plants to dehydration and low temperature. *Physiol Plant* 100: 291–296
- Close TJ, Fenton RD, Moonan F (1993) A view of plant dehydrins using antibodies specific to the carboxy terminal peptide. *Plant Mol Biol* 23: 279–286
- Danyluk J, Perron A, Houde M, Limin A, Fowler B, Benhamou N, Sarhan F (1998) Accumulation of an acidic dehydrin in the vicinity of the plasma membrane during cold acclimation of wheat. *Plant Cell* 10: 623–638
- Davies PL, Hew CL (1990) Biochemistry of fish antifreeze proteins. *FASEB J* 4: 2460–2468
- De Vries AL (1986) Antifreeze glycopeptides and peptides: Interactions with ice and water. *Methods Enzymol* 127: 293–303
- Duman JG (1994) Purification and characterization of a thermal hysteresis protein from a plant, the bittersweet nightshade *Solanum dulcamara*. *Biochim Biophys Acta* 1206: 129–135
- Duman JG, Wu DW, Olsen TM, Urrutia M, Tursman D (1993) Thermal-hysteresis proteins. *Adv Low-Temp Biol* 2: 131–182
- Dure L III (1993) Structural motifs in LEA proteins of higher plants. In: Close TJ, Bray EA (eds) *Response of Plants to Cellular Dehydration During Environmental Stress*. American Society of Plant Physiologists, Rockville, MD, pp. 91–103. ISBN 0-943088-26-7
- Egerton-Warburton LM, Balsamo RA, Close TJ (1997) Temporal expression and ultrastructural localization of dehydrins in *Zea mays* L. *Physiol Plant* 101: 545–555

- Godoy JA, Lunar R, Torres-Schumann S, Moreno J, Rodrigo RM, Pintor-Toro JA (1994) Expression, tissue distribution and sub-cellular localization of dehydrin TAS14 in salt-stressed tomato plants. *Plant Mol Biol* 26: 1921–1934
- Gong Z, Ewart KV, Hu Z, Fletcher GL, Hew CL (1996) Skin antifreeze protein genes of the winter flounder, *Pleuronectes americanus*, encode distinct and active polypeptides without the secretory signal and prosequences. *J Biol Chem* 271: 4106–4112
- Hew CH, Yang DSC (1992) Protein interaction with ice. *Eur J Biochem* 203: 33–42
- Hon WC, Griffith M, Chong P, Yang DSC (1994) Extraction and isolation of plant antifreeze proteins from winter rye (*Secale cereale* L.) leaves. *Plant Physiol* 104: 271–272
- Hon WC, 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
- Houde M, Daniel C, Lachapelle M, Allard F, LaLiberte JF, Sarhan F (1995) Immunolocalization of freezing tolerance-associated proteins in the cytoplasm and nucleoplasm of wheat crown tissues. *Plant J* 8: 583–593
- Kazuoka T, Oeda K (1994) Purification and characterization of COR85-oligomeric complex from cold-acclimated spinach. *Plant Cell Physiol* 35: 601–611
- Lin C, Thomashow MF (1992) A cold-regulated *Arabidopsis* gene encodes a polypeptide having potent cryoprotective activity. *Biochem Biophys Res Commun* 183: 1103–1108
- Mayhew M, Hartl FU (1996) Molecular chaperone proteins. In: Neidhardt F (ed.) *Escherchia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology. American Society for Microbiology, Washington, DC, pp 922–937. ISBN 1-555-81084-5.
- Muthalif MM, Rowland LJ (1994) Identification of dehydrin-like proteins responsive to chilling in floral buds of blueberry. *Plant Physiol* 104: 1439–1447
- Neven LG, Hskell DW, Guy CL, Denslow N, Klein PA, Green LG, Silverman A (1992) Association of 70-kilodalton heat-shock cognate proteins with acclimation to cold. *Plant Physiol* 99: 1362–1369
- Neuhoff VN, Arold DT, Taube D, Elhardt W (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie brilliant blue G-250 and R-250. *Electrophoresis* 9: 255–262
- Parody-Morreale A, Murphy KP, Di Cera E, Fall R, De Vries AL, Gill SJ (1988) Inhibition of bacterial ice nucleators by fish antifreeze glycoproteins. *Nature* 33: 782–783
- Pihakaski-Maunsbach K, Griffith M, Antikainen M, Maunsbach AB (1996) Immunogold localization of glucanase-like antifreeze protein in cold acclimated winter rye. *Protoplasma* 191: 115–125
- Ramagl LS, Rodriguez IV (1985) Quantification of microgram amounts of protein in two-dimensional polyacrylamide gel electrophoresis sample buffer. *Electrophoresis* 6: 559–563
- Schneider K, Wells B, Schmelzer E, Salamini F, Bartels D (1993) Desiccation leads to the rapid accumulation of both cytosolic and chloroplastic proteins in the resurrection plant *Craterostigma plantagineum* Hochst. *Planta* 189: 120–131
- Valerio PF, Kao MH, Fletcher GL (1992) Fish skin: An effective barrier to ice crystal propagation. *J Exp Biol* 164: 135–151
- Wisniewski M, Davis G (1995) Immunogold localization of pectins and glycoproteins in tissues of peach with reference to deep supercooling. *Trees* 9: 253–260
- Wisniewski M, Close TJ, Artlip TA, Arora R (1996) Seasonal patterns of dehydrins and 70-kDa heat-shock proteins in bark tissues of eight species of woody plants. *Physiol Plant* 96: 496–505
- Zamecnik J, Janacek J (1992) Interaction antifreeze proteins from cold hardened cereals with ice nucleation active bacteria. *Cryobiology* 29: 718–719