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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

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In several plant species, certain cold-regulated proteins share unique properties. These proteins are (a) heat stable and (b) hydrophilic and are related to the Group 2 late embryogenesis abundant or dehydrin family of proteins. Our previous work with sibling deciduous and evergreen peach genotypes demonstrated a correlation between the level of accumulation of certain bark proteins and cold-acclimation potential of these tissues. Here we identify a 60-kD bark protein in peach (*Prunus persica* [L.] Batsch), PCA60 ("peach cold acclimation"), that is accumulated during cold acclimation and is heat stable. Immunological studies indicated that this protein is related to the dehydrin family of proteins and accumulates at much higher levels in the bark tissues of the deciduous genotype than in the evergreen. Amino acid composition indicated that the 60-kD protein has a compositional bias for glycine (24%), glutamic acid/glutamine (11.4%), aspartic acid/asparagine (10%), and threonine (9.6%), contains relatively low levels of aromatic amino acids (phenylalanine and tyrosine), and is rich in hydrophilic amino acids. A novel characteristic of the 60-kD cold-acclimation protein is the presence of a repeating nine-amino acid sequence. A five-amino acid stretch, which is included within this repeating motif, shares striking homology with other cold-regulated proteins and dehydrins.

Physiological and molecular studies have shown that cold acclimation in higher plants induces the synthesis and/or accumulation of specific proteins as a result of altered gene expression (Guy, 1990). Although considerable effort has been directed at understanding the structure and function of these proteins in herbaceous plant species (Volger and Heber, 1975; Guy and Haskell, 1989; Hincha and Schmitt, 1992; Houde et al., 1992; Lin and Thomashow, 1992a; Lee and Chen, 1993), reports concerning deciduous fruit trees and woody plants, in general, are limited. Determining whether or not seasonal changes in protein turnover are specifically associated with cold acclimation is difficult because of the fact that overwintering deciduous perennials also enter into endodormancy during the same time as they develop cold hardiness. Therefore, the existence of sibling deciduous and evergreen peach (*Prunus persica*) genotypes (Arora et al., 1992) provides a good model system to study protein changes during cold acclimation in woody perennials that are not a direct result of endodormancy. Our previous work with these genotypes identified several proteins associated with cold acclimation and/or dormancy (Arora et al., 1992). Among these was the association between the level of accumulation of a 60-kD bark protein and cold-acclimation potential. However, the composition and function of this protein were not characterized.

It has been reported that certain cold-acclimation-induced proteins from several herbaceous and cereal plant species share the unusual property of remaining soluble upon boiling (Volger and Heber, 1975; Gilmour et al., 1992; Houde et al., 1992; Kazuoka and Oeda, 1992; Neven et al., 1993). Furthermore, a 47-kD *Arabidopsis* cor protein (Gilmour et al., 1992), a 39-kD wheat cor protein (Guo et al., 1992), and an 85-kD spinach cold-acclimation protein (CAP85) (Neven et al., 1993) are related to Group 2 LEA proteins or the dehydrin family of proteins (Close et al., 1989, 1993b). LEAs also remain soluble upon boiling, a characteristic that has been termed "heat stability," and have been suggested to play a role in water-stress tolerance (Baker et al., 1988; Close et al., 1989, 1993b).

In the present paper, we provide evidence that a 60-kD peach bark protein (which we term PCA 60 for "peach cold-acclimation" protein of 60 kD) accumulates during cold acclimation, is correlated with cold-acclimation potential of deciduous and evergreen peach genotypes, is heat stable, and is related to Group 2 LEA or the dehydrin family of proteins.

MATERIALS AND METHODS

Plant Material and Cold Hardiness Determination

Current year shoots from 3- to 4-year-old sibling deciduous and evergreen peach (*Prunus persica* [L.] Batsch) trees (Arora et al., 1992) were collected monthly at the Appalachian Fruit Research Station (Kearneysville, WV). The samples were collected in the field, packed on ice, and then processed for

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Abbreviations: cor, cold-regulated; LEA, late embryogenesis abundant; LT_{50}, temperature at which 50% injury occurred; PCA, peach cold acclimation.

cold hardiness determinations as previously described (Arora et al., 1992).

Protein Extraction and Heat-Stability Experiments

Proteins from bark tissue were extracted in borate buffer (50 mM sodium borate, 50 mM ascorbic acid, 1 mM PMSF [pH 9.0]) at 4°C as described earlier (Arora et al., 1992). For the isolation of heat-stable proteins, 3- to 4-mL aliquots of total protein extracts from each genotype were subjected to either 55, 75, or 95°C for 20 min, cooled on ice for 15 min, and centrifuged at 16,000*g* for 20 min to remove precipitated proteins; supernatant was used as the heat-stable fraction. The remaining unheated protein extract served as a control. The protein content in various fractions was measured by a modified Bradford assay (Ramagl and Rodriguez, 1985).

SDS-PAGE and Immunoblotting

Equal amounts (20 μg) of control and heat-stable protein samples were dissolved in SDS-PAGE sample buffer, and the polypeptides were fractionated by discontinuous SDS-PAGE and visualized by Coomassie stain as described earlier (Arora et al., 1992). For immunoblotting, separated proteins (3 μg) from unstained gels were electroblotted onto 0.45-μm nitrocellulose membranes (Schleicher & Schuell) using a Mini Trans-Blot Elecrophoretic Transfer Cell (Bio-Rad). Electroblotting was carried out for 1.25 h at 100 V of field intensity in Towbin buffer (25 mM Tris, 192 mM Gly, 20% [v/v] methanol [pH 8.3]; Towbin et al., 1979) at 4°C. Membranes were blocked with 1% BSA in Tris-buffered saline plus Tween 20 (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween 20) and probed at 1:1000 dilution with the antibody (kindly provided by Dr. Timothy Close) directed against a synthetic peptide of a 15-amino acid consensus sequence (EKKGIMDKIKEKLPG) that is highly conserved at the C termini of Group 2 LEA/dehydrin proteins from several monocot and dicot angiosperms, at least two gymnosperms (Close et al., 1993a), and some ferns and bryophytes (Bewley et al., 1993). Immunoreactive bands were detected by alkaline phosphatase assay using ProtoBlot Western Blot AP kit (Promega).

Protein Purification, Electroblotting, Amino Acid Composition, and Protein Sequencing

PCA60 was partially purified by fractionating the 100-mg total protein extracts by preparative, free-solution IEF using Rotofor (Bio-Rad) as described by Arora and Wisniewski (1992) and Neven et al. (1992). Proteins were focused for 4.5 h at 15 W with 4°C coolant. After the focusing, samples (20 fractions) were collected. A 5-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). Fractions were highly enriched (>80% of total protein loaded on the gel) in the 60-kD protein.

For total amino acid composition, 5-μL aliquots of fraction 15 were separated by SDS-PAGE and electroblotted onto a 0.2-μm polyvinylidene difluoride protein-sequencing membrane (Bio-Rad) as described above. Membranes were briefly stained with 0.025% Coomassie brilliant blue R-250 in 40% methanol and destained in 50% methanol. The bands corresponding to the 60-kD protein were excised and hydrolyzed in 6 N HCl. Liberated amino acids were derivatized by reaction with phenylisothiocyanate, and the resulting phenylthiocarbamyl amino acids were quantitatively determined by reverse-phase HPLC (model 130; Applied Biosystems, Foster City, CA) at the University of Michigan Protein and Carbohydrate Structure Facility.

The first attempt to sequence the 60-kD protein using excised polyvinylidene difluoride membrane blots indicated that the N terminus was blocked. Therefore, the partially purified protein (an aliquot of Rotofor fraction 15) was further purified by C_4, reverse-phase HPLC (Beckman, Fullerton, CA). Samples corresponding to the 60-kD protein peak were vacuum dried and subjected to clostripain digestion. Clostripain, a protease, cleaves peptide chains primarily at the C-terminal side of Arg residues. Soluble peptides were separated by C_{18} reverse-phase HPLC, and N-terminal sequences of individual peptides were determined by Edman degradation in a Porton PI 2090 gas-phase sequencer (Beckman). Sequencing analyses were performed at the U.S. Department of Agriculture-Agrcultural Research Service, Southern Regional Research Center (mid-south area, New Orleans, LA).

RESULTS

Seasonal Pattern of Cold Hardiness of Bark Tissues in Deciduous and Evergreen Peach

Cold hardiness determinations of bark tissues indicated an LT_{50} of about −4°C for June samples of both deciduous and evergreen peach genotypes. The bark tissues collected in January exhibited an LT_{50} of −52°C and −23°C for deciduous and evergreen peach, respectively. For more detailed reporting of seasonal changes of cold-acclimation and protein expression in these genotypes, see Arora et al. (1992). Subsequently in this report we refer to June and January samples of bark from the two peach genotypes as nonacclimated and cold-acclimated samples, respectively.

Seasonal Pattern of Proteins and Their Heat Stability and Immunoblot Analysis: Nonacclimated versus Cold-Acclimated Bark Tissues in Deciduous and Evergreen Peach

Total soluble protein content in the bark tissues of both genotypes increased from 0.33 mg g^{-1} fresh weight in the nonacclimated tissues to 3.94 and 3.24 mg g^{-1} fresh weight in the cold-acclimated tissues of deciduous and evergreen genotypes, respectively. Data from heat-coagulation experiments indicated that there was a successive increase in the coagulation of most proteins as the temperature increased from 55 to 95°C (Fig. 1). The proteins from acclimated tissues appeared to be relatively more heat stable than those from nonacclimated ones. In particular, a 60-kD polypeptide from cold-acclimated tissues appeared to be very heat stable and remained soluble even at 95°C. No proteins, however, were detected as heat stable in profiles of samples from nonacclimated tissues exposed to 95°C.

Immunoblot analysis of the heat-stable proteins from non-

acclimated and cold-acclimated tissues (Fig. 2) indicated that PCA60 was immunologically cross-reactive with the antibody raised against dehydrin protein. PCA60 was present at higher levels in deciduous trees than in evergreens. No immunoreactive band was detected in samples from nonacclimated tissues or in samples from cold-acclimated tissues that were probed with preimmune serum.

Partial Purification, Amino Acid Composition, and Partial Microsequencing of the 60-kD Peach Bark Protein (PCA60)

Protein extracts from cold-acclimated tissues were fractionated by free-solution IEF in a Rotofor apparatus. SDS-PAGE profiles of fractions 2 through 19 are presented in Figure 3. Fractions 15 and 16 were highly enriched in a 60-kD protein, having an isoelectric point of 7.3, and immunoblot analysis was conducted to confirm that this protein was indeed a dehydrin protein. The kinetics of its accumulation as a function of cold acclimation were also determined. For this purpose, SDS-PAGE and immunoblot analyses were performed on fraction 15 obtained from different samples and times.

Free-solution isoelectric-focused extracts were obtained from bark tissues of deciduous and evergreen genotypes collected in January (LT_{50} of −52 and −23°C, respectively), March (LT_{50} of −28.5 and −10°C, respectively), and June (LT_{50} of −4°C for both genotypes). The data indicated that the amount of 60-kD protein was correlated with the level of cold hardiness (LT_{50}) of the two genotypes (Fig. 4A). A significant reduction was noted in the level of 60-kD polypeptide in March compared with January samples, and this paralleled the decrease in cold hardiness. No protein bands were detected in June samples (fully deacclimated). Proteins extracted in June from both genotypes fractionated between pH 3.1 and 5.8 on the Rotofor, and a 60-kD protein was not present in these fractions (data not shown). Immunoblot analysis of the proteins from fraction 15 obtained from January, March, and June samples indicated that PCA60 was immunologically cross-reactive with antibody raised against dehydrin protein and confirmed that it was present at a higher level in deciduous than in evergreen trees.

Amino acid analysis of PCA60 indicated that it had a compositional bias for Gly (24%), Glu/Gln (11.4%), Asp/Asn (10%), and Thr (9.6%) (Table I). These four amino acids represented 55% of the total amino acids. Among the remaining amino acids, Lys was most abundant at 8%. Hydrophilic amino acids constituted about 78% of the total amino acid residues of this protein. The protein was also shown to contain relatively low levels of aromatic amino acids (Phe and Tyr).

For microsequencing analysis, PCA60 was further purified by running fraction 15, obtained from cold-acclimated tissues, through C_4 reverse-phase HPLC (see "Materials and

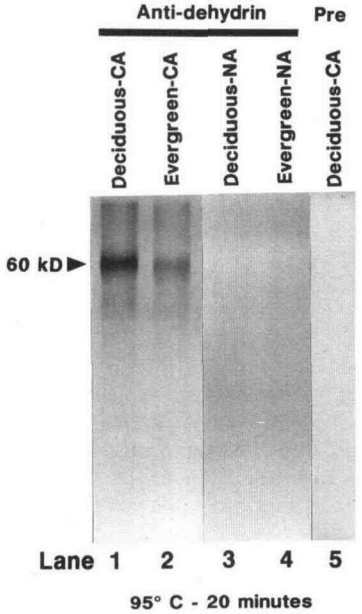

Figure 2. Western blot analysis of heat-stable bark proteins from cold-acclimated (CA) and nonacclimated (NA) tissues of sibling deciduous and evergreen peach genotypes. Each lane (1–5) was loaded with 3 μg of the heat-stable protein fraction. Lanes 1 through 4 were probed with dehydrin antiserum (Anti-dehydrin) as described in "Materials and Methods," whereas lane 5 was probed with preimmune serum (Pre).

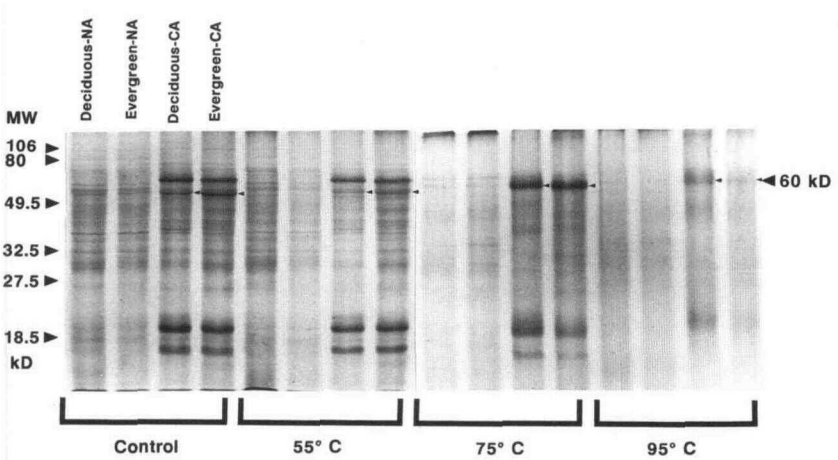

Figure 1. SDS-PAGE profile of heat-stable bark proteins extracted from nonacclimated (NA) and cold-acclimated (CA) tissues of sibling deciduous and evergreen peach genotypes. Controls are unheated samples. The protein samples (four lanes each) corresponding to 55, 75, and 95°C heat treatments are loaded in the same sequence as for the control. Protein (20 μg) was loaded in each lane. Molecular masses (MW) of protein standards are indicated to the left.

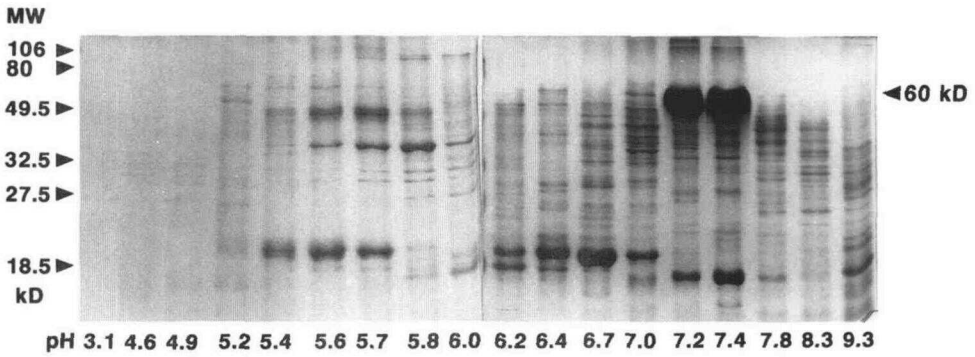

Figure 3. SDS-PAGE analysis of Rotofor fractions (lanes 2–19) containing soluble bark proteins from cold-acclimated tissues of the deciduous peach genotype. Fractions were obtained by free-solution IEF as described in "Materials and Methods." Eight microliters of sample was loaded in each lane. Fractions 15 and 16 are highly enriched with a 60-kD protein (arrowhead). The pH of each fraction, which corresponds to the isoelectric point of proteins in that fraction, is indicated at the bottom. Molecular masses (MW) of protein standards are indicated on the left.

Methods"). Partial cleavage of purified 60-kD protein by clostripain produced three peptides. Gas-phase sequencing demonstrated that these peptides contained a portion of nine amino acids that was almost identical in the three fragments (Table II). Hence, if we assume that PCA60 is truly a homogeneous protein, the nine-amino acid stretch was repeated at least three times in the sequence (being present once in each of three fragments). A five-amino acid stretch of this nine-amino acid-long repeating motif showed sequence homology to repeating motifs in dehydrins, drought-induced proteins, and cold-acclimation proteins from other plant species.

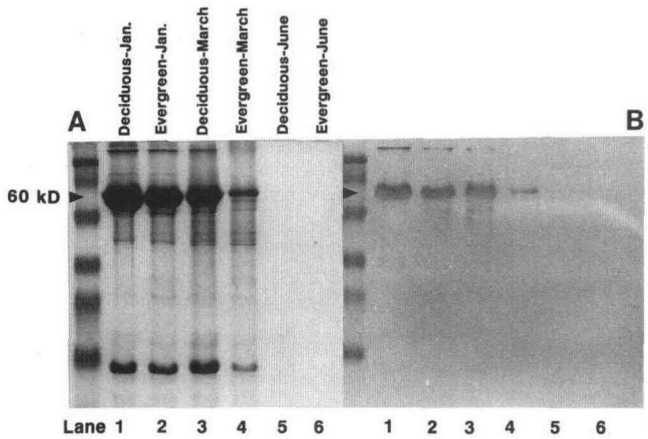

Figure 4. A, SDS-PAGE analysis (lanes 1–6) of Rotofor fraction 15 (enriched in the 60-kD bark protein as indicated by arrowhead) containing proteins extracted from deciduous and evergreen peach genotypes in January (Jan.), March, and June. Eight microliters of protein sample was loaded on each lane. Molecular mass markers are on the left. B, Western blot analysis (lanes 1–6) of proteins in A, using anti-dehydrin antiserum. One microliter of protein sample was loaded on each lane and electroblotted on nitrocellulose membrane as described in "Materials and Methods." The arrowhead indicates the presence of dehydrin protein.

DISCUSSION

Heat Stability and Expression of Dehydrin-Like, 60-kD Protein during Cold Acclimation

We have previously reported that accumulation of a 60-kD protein is associated with the capacity of peach bark tissues to develop freezing tolerance (Arora et al., 1992), but its composition and function were not characterized. Data from the present study indicate that the 60-kD peach bark protein (PCA60) is heat stable (Fig. 1) and immunologically related to the Group 2 LEA/dehydrin family of proteins (Fig. 2). A recent study by Close et al. (1993a) has provided evidence for the presence of heat-stable dehydrin proteins in the seeds of woody perennials such as pinon pine (*Pinus edulis*) and ginkgo (*Ginkgo biloba*).

Close et al. (1993a) reported the presence of dehydrins ranging from 15 to 120 kD in several species of monocots and dicots and two gymnosperms. The majority of the dehydrins detected were in the range of 15 to 40 kD; however,

Table I. *Total amino acid composition of PCA60*

Amino Acid	Mol (%)
Ala	5.7
Arg	1.8
Asp	10.0
Glu	11.4
Gly	24.0
His	6.2
Ile	1.2
Leu	4.2
Lys	8.0
Met	0.7
Phe	0.5
Pro	5.9
Ser	2.3
Thr	9.6
Tyr	4.7
Val	3.9

Table II. *Amino acid-repeating motifs in PCA60 and comparison with other stress-related and dehydrin proteins*

Plant	Polypeptide		Sequence of Repeats	Reference
Peach	60 kD	1	RLPGGQKDDQYL	
		2	RLPGGQNVDPTTGPYGGGGAAG	
		3	RLPIGQKVD	
Barley	B8	1	KLPGGAH	Close et al. (1989)
		2	KLPGGQH	
	B9	1	KLPGGAH	
		2	KLPGGQH	
Spinach	CAP	85	KLPG-QH	Neven et al. (1993)
Wheat	COR	39	KLPGG-H	Guo et al. (1992)
	WCS	120	KLPGGHGDHQQTGGT	Houde et al. (1992)
Craterostigma plantagineum	C6-19	1	KLPGG-H	Piatkowski et al. (1990)
		2	KLPGGQH	
Consensus[a]			R/KLPGGQ	

[a] Conservative substitution is K = R.

a dehydrin at 60 kD was also observed in the dehydrated shoots of rice and barley. In the present study, the immunoblot analyses of unheated extracts of proteins from cold-acclimated tissues of the two genotypes revealed at least three other bands of dehydrin-like proteins at about 38 to 50 kD, in addition to the major protein band at 60 kD (data not shown). No immunoreactive bands were detected in unheated protein samples from nonacclimated tissues. In the heated extracts from acclimated tissues, however, only PCA60 was recognized by the dehydrin antibody (Fig. 2). These results suggest that the expression of polypeptides belonging to the dehydrin family of proteins may be a general response during cold acclimation in peach and the heat stability may not be a general property of all dehydrin-like proteins in the bark tissues of peach. More detailed studies are needed to support or disprove this conclusion in a definitive manner.

LEA proteins, which represent a diverse group, are synthesized during late embryogenesis just prior to seed desiccation (Galau et al., 1986) and, in some cases, in response to water stress (Close et al., 1989). LEA proteins are thought to act as water-stress protectants (Baker et al., 1988; Close et al., 1989). The potential significance of LEAs in cold acclimation lies in the fact that plant cells undergo dehydration during freezing stress due to the presence of ice in extracellular spaces (Levitt, 1980). Thus, responses evoked because of water stress may also be involved in freezing tolerance mechanisms. Our results of the accumulation of a Group 2 LEA/dehydrin protein during cold acclimation and many recent findings indicating a relatedness of several cor proteins with Group 2 LEAs or dehydrins based on their heat stability (Lin et al., 1990; Gilmour et al., 1992; Houde et al., 1992; Neven et al., 1993) and DNA sequence (Gilmour et al., 1992; Guo et al., 1992; Houde et al., 1992; Neven et al., 1993) are consistent with this view. It is noteworthy that PCA60 was present at relatively higher levels in cold-acclimated tissue of deciduous trees than that in evergreens (Figs. 2 and 4). This is in accordance with our earlier study of the seasonal pattern of SDS-PAGE profiles of bark proteins from these two genotypes and provides further evidence that this protein may play a role in conferring freezing tolerance in peach trees. Recently, based on an immunogenic reaction, we detected a 60-kD, dehydrin-like protein in the leaves of deciduous and evergreen peach. Preliminary results indicated that it was present at higher levels in cold-acclimated leaf tissue than in nonacclimated ones (R. Arora and M.E. Wisniewski, unpublished results).

Amino Acid Composition

The PCA60 investigated in the present study is Gly rich, low in aromatic amino acids, and rich in hydrophilic amino acids (Table I). Similar characteristics have been observed for dehydrins from barley and corn (Close et al., 1989), two high mol wt cold-acclimation proteins from spinach (Guy and Haskell, 1989), a 200-kD cold-acclimation-induced protein from wheat (Ouellet et al., 1993), and heat-stable proteins from aleurone cells of barley (Jacobsen and Shaw, 1989). Houde et al. (1992) suggested that high Gly content may confer high flexibility to proteins in terms of stretching, bending, and expanding, a property that could be useful to protect cellular structures against freeze-induced dehydration. In addition to Gly, PCA60 is also rich in Glu/Gln, Asp/Asn, and Thr. These amino acids are also abundant in at least three cold-acclimation proteins (160, 85, and 79 kD) in spinach (Guy and Haskell, 1989), dehydrins from various cereals (Close et al., 1989), and a 200-kD cold-acclimation-induced protein in wheat (Ouellet et al., 1993). Dehydrins have also been shown to contain relatively high amounts of Lys (Close et al., 1989), a characteristic also shared by cold-acclimation-induced proteins in spinach (Neven et al., 1993) and *Arabidopsis* (Gilmour et al., 1992). Consistent with these observations, we also noted a high amount of Lys in PCA60 (Table I).

The PCA60 is also specifically rich in those amino acids that are strongly hydrophilic on the Kyte and Doolittle (1982) hydropathy scale and therefore might be expected to be highly hydrophilic. This is in accordance with the hydrophilic

nature of other cor/cold-acclimation proteins (Volger and Heber, 1975; Gilmour et al., 1992; Guo et al., 1992; Houde et al., 1992; Lin et al., 1992a, 1992b; Neven et al., 1993) and the dehydrin family of proteins. The expression of a hydrophilic protein during cold acclimation may have a significance in terms of its capacity to trap enough water inside the cell to prevent local dehydration that may occur during extracellular freezing. Alternatively, Houde et al. (1992) have suggested that high hydrophilicity may induce hydrogen bonding with nascent ice crystals, which may modify the structure or propagation of ice crystals and reduce intracellular freezing damage.

Sequence Comparison

Preliminary attempts to obtain the N-terminal sequence of the 60-kD protein were unsuccessful because of an apparent blockage of N termini (data not shown). A partial sequence obtained from three fragments generated by partial clostripain digestion of the PCA60 revealed an almost identical sequence of nine amino acids common to all three fragments. This suggests that PCA60 contains repeated motifs in its primary structure. Repeated elements are a characteristic of other stress-related proteins (Piatkowski et al., 1990; Guo et al., 1992; Houde et al., 1992; Neven et al., 1993) and dehydrins (Close et al., 1989). As in several cor proteins (Gilmour et al., 1992; Guo et al., 1992; Houde et al., 1992; Nevén et al., 1993) and dehydrins (Close et al., 1989), the repeating motif of PCA60 is predominantly hydrophilic. Comparison with sequences of other stress-related proteins revealed that a five-amino acid stretch of the repeating motifs in PCA60 was homologous to the repeating motifs in a number of other cold-acclimation- and dehydrin proteins (Table II). Although a partial amino acid sequence must be interpreted with caution, our results indicate that PCA60 may be closely related to other cor proteins and/or the dehydrin family of proteins from herbaceous and cereal plants.

In summary, our results provide some evidence for the view of earlier studies (Gilmour et al., 1992; Neven et al., 1993) that freezing and dehydration tolerance involve related mechanisms that include expression of LEA or dehydrin proteins. The biochemical or biophysical role of dehydrin-like proteins during cold acclimation is not known; however, a recent study by Lin and Thomashow (1992a) demonstrated that COR15, a cold-regulated, heat-stable, and hydrophilic polypeptide encoded by the *Arabidopsis cor 15* gene, had potent cryoprotective activity. Further experiments are needed to determine whether or not PCA60 exhibits cryoprotective activity.

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