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Purification and Properties of *Arabidopsis thaliana COR* (Cold-Regulated) Gene Polypeptides COR15am and COR6.6 Expressed in *Escherichia coli*[1]

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Arabidopsis thaliana **cold-regulated genes *COR15a* and *COR6.6* encode 15- and 6.6-kD polypeptides, respectively. The COR15a polypeptide is known to be targeted to chloroplasts and, during import, to be processed to a 9.4-kD polypeptide designated COR15am. The COR6.6 polypeptide is thought to be located in the cytosol. The coding sequences for COR15am and COR6.6 were fused to the bacteriophage T7 promoter and expressed in *Escherichia coli*. The recombinant polypeptides COR15am^r and COR6.6^r were purified to near homogeneity using a combination of ammonium sulfate fractionation, ion-exchange chromatography, and adsorption chromatography on hydroxyapatite. COR15am^r and the major species of COR15am in chloroplasts co-migrated on both two-dimensional O'Farrell gels and nondenaturing polyacrylamide gels. These data corroborate the site of COR15a processing and indicate no difference in charge or quaternary structure between COR15am^r and the major species of COR15am in plants. In contrast, the migration patterns of COR6.6^r and COR6.6 on two-dimensional gels suggest that a considerable portion of the COR6.6 population in plants is modified. In the accompanying papers (M.S. Webb, S.J. Gilmour, M.F. Thomashow, P.L. Steponkus [1996] Plant Physiology 111: 301–312; M. Uemura, S.J. Gilmour, M.F. Thomashow, P.L. Steponkus [1996] Plant Physiology 111: 313–327), the effects of COR15am^r and COR6.6^r on the cryostability and lyotropic phase behavior of liposomes are examined.**

Changes in gene expression occur during cold acclimation, the process whereby plants increase in freezing tolerance in response to low, nonfreezing temperatures (Guy, 1990; Thomashow, 1990, 1993). It has been widely speculated that the enhancement of freezing tolerance that occurs during cold acclimation is due, in part, to the action of cold-regulated genes. However, there is little direct evidence to support this notion. Until recently, the strongest case in favor of this hypothesis had come from the work of Heber and colleagues (Volger and Heber, 1975; Hincha et al., 1989, 1990). These investigators have presented data indicating that cold-acclimated spinach and cabbage, but not nonacclimated plants, accumulate proteins that have potent cryoprotective activity in vitro; the proteins are reported to be about 10^4 times more effective than Suc (on a molar basis) in protecting isolated thylakoids against freeze-thaw damage. The mechanism of this protection is not well understood, but appears to involve reducing membrane permeability during freezing and increasing membrane expandability during thawing (Hincha et al., 1990).

The hypothesis that certain cold-regulated genes encode cryoprotective proteins is attractive. Unfortunately, work on the putative cryoprotective proteins of cabbage and spinach has been stymied by their recalcitrance to purification (Hincha et al., 1990). Early work (Volger and Heber, 1975) resulted in highly enriched protein preparations, but subsequent efforts (Hincha et al., 1989, 1990) have not yet yielded purified proteins. Indeed, specific proteins with cryoprotective activity have not been conclusively identified in cabbage and spinach, and genes encoding the putative cryoprotective polypeptides have not been isolated.

Arabidopsis thaliana, like cabbage and spinach, alters gene expression and increases in freezing tolerance in response to low, nonfreezing temperatures (Gilmour et al., 1988). Available data suggest that certain *A. thaliana COR* (cold-regulated) genes, including *COR15a* (Lin and Thomashow, 1992) and *COR6.6* (Gilmour et al., 1992), may encode analogs of the putative cabbage and spinach cryoprotective polypeptides. *COR15a* and *COR6.6* encode polypeptides of 15 and 6.6 kD, respectively. COR15a has a signal sequence that directs import of the polypeptide into chloroplasts; during the translocation process, the signal peptide of COR15a is cleaved off the polypeptide, resulting in production of the 9.4-kD mature polypeptide, COR15am (Lin and Thomashow, 1992). COR6.6 has no obvious targeting signals and is likely to be located in the cytoplasm (Gilmour et al., 1992). COR15am and COR6.6 share a number of distinctive characteristics with the spinach and cabbage proteins: they accumulate in response to low, nonfreezing temperatures; they remain soluble upon boiling in aqueous solution; they have low molecular masses (less than 20 kD); they are hydrophilic; they are rich in Ala and Gly; and they have very low A_{280}. Moreover, constitutive expression of *COR15a* enhances the freezing tolerance of chloroplasts in

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Abbreviation: NEPHGE, nonequilibrium pH gel electrophoresis.

nonacclimated *A. thaliana* plants (Artus et al., 1994; N.N. Artus, S.J. Gilmour, C. Lin, M.F. Thomashow, unpublished data).

The goal of the research presented in this and the accompanying papers (Uemura et al., 1996; Webb et al., 1996) was to examine properties of COR15am and COR6.6 that potentially relate to roles in freezing tolerance, including their effects on the cryostability and lyotropic phase behavior of liposomes. In this paper we specifically report on expression of these COR polypeptides in *Escherichia coli*, their purification to near homogeneity, and comparisons with the corresponding polypeptides produced in plants.

MATERIALS AND METHODS

Plasmid Construction

The coding sequences for COR15am and COR6.6, obtained from plasmids pLCT10 (Lin and Thomashow, 1992) and pHH29 (Hajela et al., 1990), respectively, were amplified by PCR (Innis and Gelfand, 1990). The specific primers used for COR15am amplification were TCTCCATGGCTAAAGGTGACGGC (5′ primer) and ACGGTGTTTCATCCCTAGGTGG (3′ primer), and those used for COR6.6 were AAACCATGGCAGAGACCAAC (5′ primer) and AGTTGTTCATCCCTAGGCTC (3′ primer). These primers introduced an *Nco*I site at the initiating ATG codon of each polypeptide and a *Bam*HI site immediately after each stop codon (see Fig. 1). Because COR15am does not normally possess an N-terminal Met codon, an N-terminal ATG was introduced along with the *Nco*I site. The amplified DNA fragments were ligated into the *Nco*I and *Bam*HI sites of the pET-9d bacterial expression vector (Novagen, Madison, WI). The resulting plasmids were transformed into *Escherichia coli* strain BL21(DE3)plysS (Novagen) using standard procedures (Sambrook et al., 1989).

Protein Extraction and Purification from *E. coli*

Recombinant COR15am and COR6.6, designated COR15am^r and COR6.6^r, respectively, were expressed in *E. coli* as recommended by the supplier of the expression vector (Novagen). Briefly, cells were grown in 500-mL batches of Luria-Bertani medium (Sambrook et al., 1989) to mid- to late-logarithmic phase, and protein expression was induced by the addition of 0.4 mM isopropylthio-β-galactoside. The cultures were incubated for an additional 3 h prior to extraction.

Cells synthesizing either COR15am^r or COR6.6^r were disrupted using a French press (SLM Aminco, Rochester, NY) at 16,000 p.s.i., and the extracts were clarified by centrifugation at 10,000*g* for 10 min. COR15am^r was purified by a combination of ammonium sulfate fractionation and anion-exchange chromatography. Specifically, the precipitate from a 70 to 100% saturated ammonium sulfate fractionation of the crude cell extract was collected by centrifugation, dialyzed against 25 mM potassium phosphate, pH 7.0, and applied to a DEAE-cellulose column (2.5 × 15 cm) that had been equilibrated with 25 mM potassium phosphate, pH 7.0. The COR15am^r polypeptide was then eluted using a 200-mL gradient of 0 to 50 mM NaCl in the same buffer. The fractions containing COR15am^r were pooled, concentrated using an ultrafiltration-stirred cell (Amicon, Beverly, MA) fitted with a 3000-kD molecular mass cutoff filter, and dialyzed against distilled water prior to use.

COR6.6^r was purified by a combination of ammonium sulfate fractionation, anion-exchange chromatography, and adsorption to hydroxyapatite. Specifically, the precipitate from a 50 to 70% saturated ammonium sulfate fractionation of the crude cell lysate was collected by centrifugation, dialyzed with 25 mM potassium phosphate, pH 7.0, and applied to a DEAE-cellulose column (2.5 × 15 cm) that had

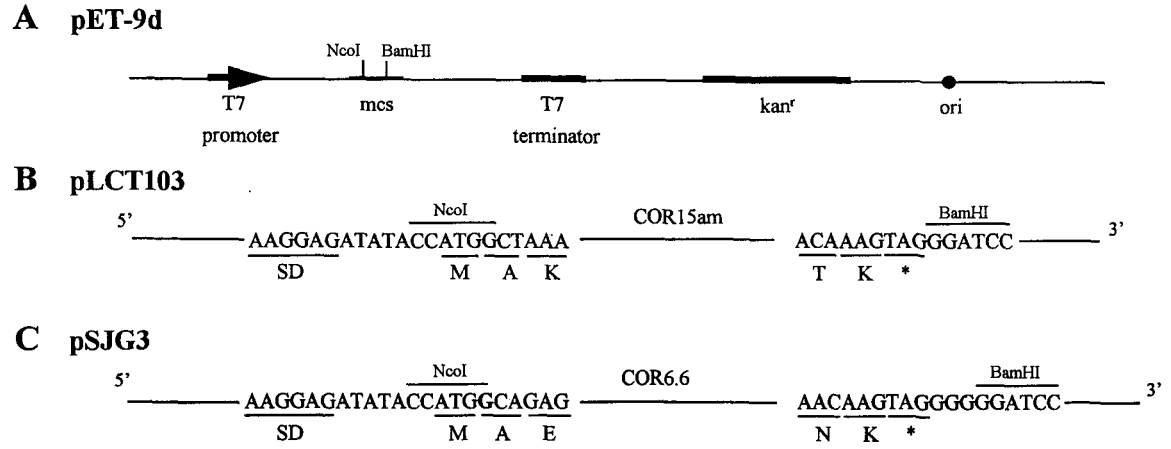

Figure 1. Plasmid constructs for synthesis of COR6.6^r and COR15am^r in *E. coli*. A, Diagram of the pET-9d expression vector. B, The 5′ and 3′ regions of COR15am^r in pLCT103. The first amino acids in COR15am^r are MAK, whereas in COR15am they are AK (see text). C, The 5′ and 3′ regions of COR6.6^r in pSJG3. The G shown in boldface (the last position in the *Nco*I recognition site) was altered from a T in the original *COR6.6* sequence to allow introduction of the *Nco*I site. The first three amino acids in COR6.6^r are MAE, whereas in COR6.6 they are MSE (see text). mcs, Multiple cloning site; SD, Shine-Dalgarno ribosome-binding site.

been equilibrated against 25 mM potassium phosphate, pH 7.0. The COR6.6^r polypeptide was eluted from the column with other nonbinding proteins by washing with 25 mM potassium phosphate, pH 7.0. The protein in this fraction was then concentrated using the ultrafiltration-stirred cell (Amicon) fitted with a 3000-kD molecular mass cutoff filter. The protein fraction was dialyzed against 1 mM potassium phosphate, pH 7.0, and applied to a hydroxyapatite column (2.5 × 5 cm) that had been equilibrated with 1 mM potassium phosphate, pH 7.0. The column was washed with this buffer until no additional protein eluted. The bound COR6.6^r was then eluted with a 200-mL gradient of unbuffered 10 to 500 mM NaCl (Gorbunoff, 1985). The fractions containing COR6.6^r were pooled, concentrated using the ultrafiltration-stirred cell, and dialyzed against distilled water prior to use.

Carbohydrate, Nucleic Acid, Amino Acid, and Molecular Mass Analysis

Total carbohydrate was determined by the phenol-sulfuric acid assay (Ashwell, 1966) with Glc serving as the standard. Nucleic acid content was estimated by obtaining UV spectra from 205 to 340 nm using a Gilford Response (Gilford, Oberlin, OH) spectrophotometer. Amino acid analysis was performed on a Waters System PICO-TAG amino acid analyzer at the Michigan State University Macromolecular Structure Facility. Molecular mass was determined by matrix-assisted laser desorption ionization MS (Hillenkamp and Karas, 1990) at the Michigan State University-National Institutes of Health Spectrometry Facility.

Plant Protein Extraction

Arabidopsis thaliana plants were grown and cold acclimated as described previously (Gilmour et al., 1988). Leaf material (0.1 g) was extracted in a 1.5-μL polypropylene homogenizer (Kontes, Vineland, NJ) in 400 μL of 50 mM Hepes, pH 7.2, with 0.01 g of polyvinylpolypyrrolidone. In some cases, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (Sigma) and 3 μg/mL pepstatin A (Sigma) were used as protease inhibitors in the extraction buffer. After two 10-min centrifugations at full speed in a microcentrifuge (Baxter, McGaw Park, IL), the supernatant was either acetone precipitated or run directly on polyacrylamide gels. Protein determinations were carried out using the dye-binding method of Bradford (1976) with BSA as the standard.

Chloroplast Isolation

Chloroplasts were isolated from cold-acclimated Arabidopsis leaves that had been placed in the dark for 24 h to reduce starch accumulation. A modification of the procedure of Perry et al. (1991) was used. Leaves were homogenized in a buffer consisting of 0.33 M sorbitol, 50 mM Hepes (pH 7.5), 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 2 mM EDTA using a Tissumizer (Tekmar, Cincinnati, OH). The homogenate was filtered through two layers of Miracloth (Calbiochem) and centrifuged at 4000 rpm for 5 min in a Sorvall HB4 rotor. The precipitate, which is enriched for chloroplasts, was resuspended in homogenization buffer without sorbitol to rupture the chloroplasts. Membranes were removed from the soluble protein extract by centrifugation at 93,792*g* for 1 h in a Sorvall RC70 ultracentrifuge using a fixed-angle rotor. Where necessary, the supernatant was concentrated in a stirred cell concentrator (Amicon).

Gel Electrophoresis and Immunoblot Analysis

One-dimensional SDS-Tricine gels were run as described (Schägger and von Jagow, 1987). Standard two-dimensional O'Farrell gels (O'Farrell, 1975) and NEPHGE (O'Farrell et al., 1977) were carried out as previously described except that SDS-Tricine gels were used for the second dimension. Nondenaturing gels using a 5 to 15% polyacrylamide gradient were run according to Laemmli (1970) but without the addition of SDS. Proteins in the one- and two-dimensional gels were visualized by staining with Coomassie blue. Immunoblot transfers to nitrocellulose were performed (Towbin et al., 1979), and the blots were probed with antisera produced in rabbits against the recombinant proteins. The protein was visualized using alkaline phosphatase conjugated to protein A (Sigma) (Blake et al., 1984).

Densitometry

Dried Coomassie blue-stained gels were scanned on a Molecular Dynamics (Sunnyvale, CA) densitometer with Image Quant software at the Michigan State University-Department of Energy Plant Research Laboratory Sequencing Facility.

RESULTS

Synthesis of COR15am and COR6.6 in *E. coli*

COR15am and COR6.6 were synthesized in *E. coli* by placing the coding sequences for each polypeptide under control of the bacteriophage T7 promoter in the pET-9d expression vector (Fig. 1). Each construct required introduction of an *Nco*I restriction site at an initiating ATG Met codon (see "Materials and Methods"). In the case of COR15am, an initiating ATG codon had to be introduced immediately upstream (5′) of the internal Ala residue predicted to be at the N terminus of the mature, processed COR15am polypeptide (Lin and Thomashow, 1992). Other than this, the amino acid sequence of COR15am was unaltered. With COR6.6, introduction of the *Nco*I restriction site at the N-terminal Met resulted in the second amino acid of the polypeptide, a Ser, being converted to an Ala. The recombinant polypeptides were designated COR15am^r and COR6.6^r. The final plasmids, pLCT103 and pSJG3, produced COR15am^r and COR6.6^r, respectively, at relatively high levels (Fig. 2A). The proteins were present in the soluble fraction of lysed cells (no inclusion bodies were detected) and remained soluble upon boiling (Fig. 2B).

Purification of COR15am^r and COR6.6^r

COR15am^r and COR6.6^r were purified from *E. coli* cell lysates by a combination of ammonium sulfate fraction-

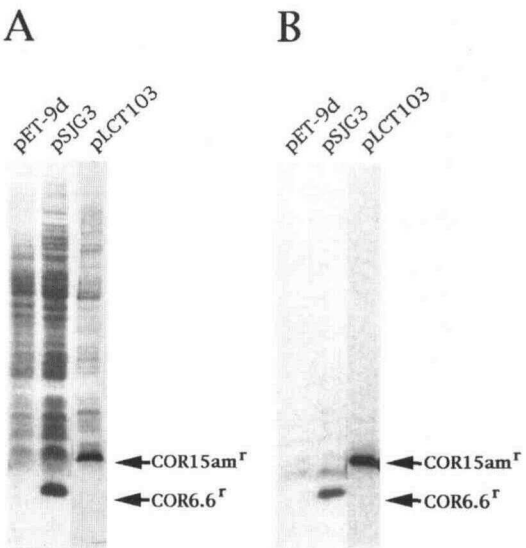

Figure 2. Synthesis of COR15am^r and COR6.6^r in *E. coli*. A, Crude cell lysates (about 100 μg of protein) prepared from *E. coli* strains carrying the indicated plasmids. B, Boiling-soluble polypeptides (about 20 μg) prepared from *E. coli* strains carrying the indicated plasmids. Crude cell lysates were prepared and fractionated on SDS-Tricine gels as described in "Materials and Methods." Boiling-soluble polypeptides were prepared by heating crude cell extracts in a boiling water bath for 10 min and removing the coagulated insoluble protein by sedimentation in a microcentrifuge for 10 min at full speed. Proteins were visualized by staining gels with Coomassie blue.

ation, ion-exchange chromatography, and adsorption chromatography on hydroxyapatite (Fig. 3). A boiling-enrichment step was not used in the purification procedure to minimize the chance of denaturing the recombinant polypeptides. The COR15am^r and COR6.6^r polypeptides accounted for greater than 90% of the protein in the final preparations as judged by densitometric analysis of Coomassie blue-stained gels. Total carbohydrate and nucleic acid typically accounted for less than 1% (w/w) of the purified samples.

Properties of Purified Polypeptides

The purified COR15am^r and COR6.6^r polypeptides migrated on Tricine SDS-PAGE gels with apparent molecular masses of 8.4 and 5.1 kD, respectively. The actual molecular masses of COR15am^r and COR6.6^r, determined by matrix-assisted, laser desorption ionization MS, were 9357 and 6403 D, respectively. These values were 127 and 132 mass units, respectively, less than expected from the amino acid sequences of the polypeptides deduced from nucleic acid sequence analysis. These data suggested that the N-terminal Met residues had been removed from the polypeptides, as often occurs in *E. coli* (see Ben-Bassat et al., 1987, and refs. cited therein). Indeed, amino acid composition analysis indicated that neither protein contained Met residues (data not shown).

Purified samples of COR15am^r and total soluble proteins prepared from chloroplasts of cold-acclimated and nonacclimated plants were fractionated on two-dimensional O'Farrell gels, and the COR15am^r and COR15am polypeptides were detected by immunoblot analysis (Fig. 4). The results indicated that COR15am^r migrated as a single major form (Fig. 4A). The samples of COR15am prepared from chloroplasts of cold-acclimated plants produced a major form and two minor ones, the latter two being located just to the left and just below the major form (Fig. 4B). The lower-molecular-mass minor form is probably a COR15am degradation product because addition of purified COR15am^r to total protein extracts prepared from nonacclimated plants yielded two polypeptides, one that co-migrated with COR15am^r and one that migrated just below COR15am^r on SDS-PAGE (not shown). COR15am was not detected in the protein samples prepared from chloroplasts of nonacclimated plants (not shown). When purified COR15am^r was mixed with the chloroplast protein samples from cold-acclimated plants and the samples were fractionated on two-dimensional O'Farrell gels, one major spot was detected, indicating that COR15am^r co-migrated with the major form of COR15am (Fig. 4C). Thus, the size and charge of the COR15am^r polypeptide appeared to be the same as those of the major species of COR15am present in the chloroplasts of cold-acclimated plants. The data also indicated that the putative consensus processing site for the chloroplast-targeting sequence (Lin and Thomashow, 1992) was the primary, if not sole, site used during import of COR15a.

Additional experiments (Fig. 5) indicated that purified COR15am^r co-migrated on nondenaturing polyacrylamide

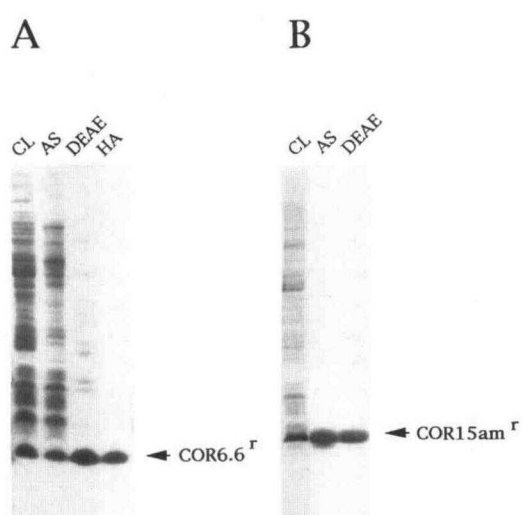

Figure 3. Steps in purification of recombinant COR polypeptides. A, COR6.6^r. B, COR15am^r. Crude cell lysates of *E. coli* carrying either pSJG3 (A) or pLCT103 (B) were prepared and fractionated as described in "Materials and Methods." The polypeptides present at each stage of the purification process were determined by fractionating samples on SDS-Tricine gels and staining the polypeptides with Coomassie blue. The steps indicated are: CL, crude lysates (approximately 200 and 100 μg of protein in A and B, respectively); AS, ammonium sulfate fraction (approximately 100 and 30 μg of protein in A and B, respectively); DEAE, DEAE fraction (approximately 50 and 20 μg of protein in A and B, respectively); and HA, hydroxyapatite fraction (approximately 20 μg of protein).

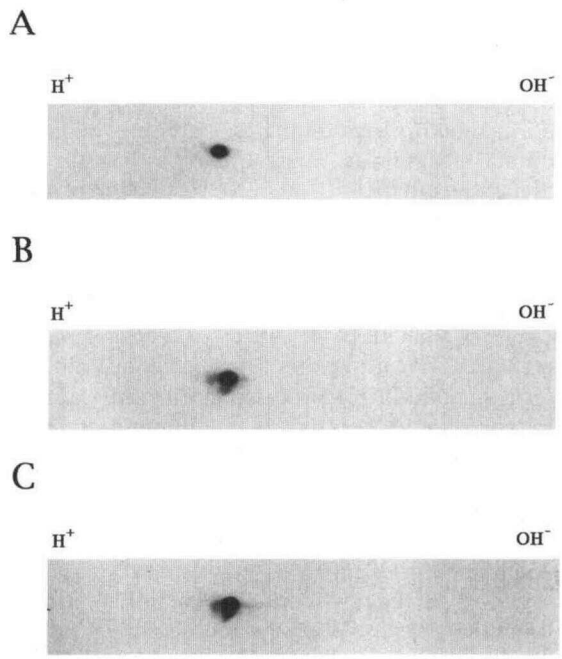

Figure 4. Migration of COR15am^r and COR15am on two-dimensional O'Farrell gels. A, Purified sample of COR15am^r (50 ng). B, Soluble chloroplast proteins from plants cold acclimated for 7 d (10 μg). C, Mixture of samples used in A and B. Protein samples were prepared and fractionated on two-dimensional O'Farrell gels and COR15am^r/COR15am was detected by immunoblot analysis as described in "Materials and Methods." The pH gradient ranged from approximately pH 4.5 to 7.0.

gels, with the major species of COR15am present in chloroplasts of cold-acclimated plants (the nature of the minor bands that reacted with the COR15am antiserum is not known). These data suggest that COR15am is not an integral member of a heteromeric protein. However, both COR15am^r and COR15am migrated with apparent molecular masses of about 70 kD. Whether this migration is due to COR15am^r and COR15am forming multimers or results from aberrant mobility of the polypeptides, perhaps due to their low pI (deduced from the amino acid sequence to be 4.6), remains to be determined.

Recombinant COR6.6^r has a predicted pI of approximately 9.9 and thus would not be expected to focus in the first dimension of a standard two-dimensional O'Farrell gel. Samples of COR6.6^r, therefore, were fractionated on two-dimensional NEPHGE gels and visualized by immunoblotting (Fig. 6A). One primary form was observed. In contrast, multiple forms were detected in many samples of total soluble protein prepared from cold-acclimated plants (Fig. 6B). Mixing experiments indicated that the COR6.6^r polypeptide co-migrated with the most basic (rightmost) form in the plant protein sample (data not shown). These results indicated that a considerable portion (perhaps half) of the plant COR6.6 was more acidic than COR6.6^r. Indeed, whereas little if any of the COR6.6^r polypeptide entered the focusing gel on standard two-dimensional O'Farrell gels (Fig. 7A), a number of forms were detected with protein samples from cold-acclimated plants (Fig. 7B). These polypeptides were not observed with protein samples prepared from nonacclimated plants (not shown). They were, however, observed in nonacclimated transgenic plants that constitutively expressed the *COR6.6* gene, indicating that the polypeptides were indeed derived from the COR6.6 protein (S.J. Gilmour, unpublished data).

DISCUSSION

The *COR15a* and *COR6.6* genes of Arabidopsis (Hajela et al., 1990) and corresponding homologs in *Brassica* spp. (Orr et al., 1992; Weretilnyk et al., 1993) are among the most highly expressed cold-regulated genes of these plant species. The function(s) of these genes is not yet known. However, we have speculated (Lin and Thomashow, 1992) that they might encode cryoprotective proteins that reduce cellular damage caused by the severe dehydration associated with freezing. This suggestion was based on the fact that COR15am and COR6.6 share a number of distinctive characteristics with the putative cryoprotective proteins of spinach and cabbage (Volger and Heber, 1975; Hincha et al., 1990) and that *COR15a* and *COR6.6* transcripts accumulate in response to drought (Hajela et al., 1990). It is also of interest that the COR15am and COR6.6 polypeptides have a number of distinctive properties in common with LEA (late-embryogenesis abundant) proteins (Dure et al., 1989), polypeptides suggested to have a role in water-stress tolerance (Baker et al., 1988). The similarities between these COR polypeptides and LEA polypeptides include boiling solubility (the polypeptides remain soluble upon boiling), hydrophilicity, repeated amino acid sequence motifs, and a

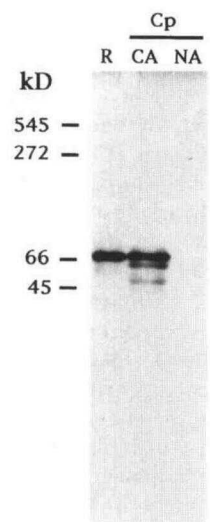

Figure 5. Migration of COR15am^r and COR15am on nondenaturing polyacrylamide gels. Protein samples are: R, recombinant COR15am^r (300 ng); Cp/CA, protein from chloroplasts of plants cold acclimated for 4 weeks (20 μg); Cp/NA, protein from chloroplasts of nonacclimated plants (20 μg). Protein samples were prepared and fractionated on 5 to 15% polyacrylamide nondenaturing gels, and COR15am^r/COR15am was detected by immunoblot analysis as described in "Materials and Methods." Size markers in kD are as indicated.

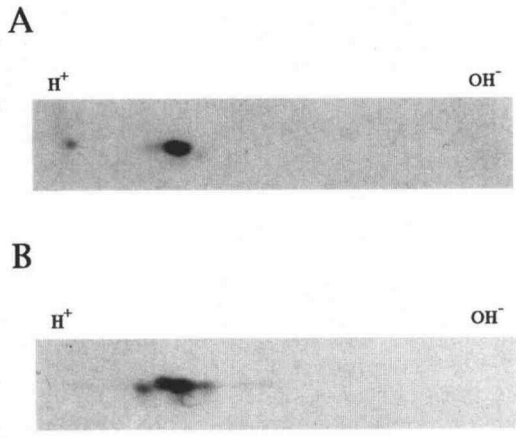

Figure 6. *Migration of COR6.6^r and COR6.6 on two-dimensional NEPHGE gels. A, Purified sample of COR6.6^r (300 ng). B, Total soluble proteins from plants cold acclimated for 4 weeks (200 μg). Protein samples were prepared and fractionated on two-dimensional NEPHGE gels, and COR6.6^r/COR6.6 was detected by immunoblot analysis as described in "Materials and Methods." The pH gradient ranged from approximately pH 7.5 to 9.5.*

low degree of amino acid sequence identity (specifically between the COR polypeptides and the group III family of LEA proteins) (Gilmour et al., 1992; Wilhelm and Thomashow, 1993).

One approach that we have taken to test the "cryoprotection hypothesis" is to express the *COR15a* and *COR6.6* genes constitutively in Arabidopsis and assess the effects that this has on freezing tolerance. The results indicate that constitutive expression of the COR15am polypeptide enhances the freezing tolerance of chloroplasts in nonacclimated plants (Artus et al., 1994; N.N. Artus, S.J. Gilmour, C. Lin, M.F. Thomashow, unpublished data). Whether this effect is due to COR15am acting directly as a cryoprotectant, however, is not known. The work presented in this and accompanying papers (Uemura et al., 1996; Webb et al., 1996) was conducted, in part, to address this question. We were particularly interested in determining whether COR15am and/or COR6.6 affect the freezing behavior of membranes. This is of interest because membranes have been shown to be a primary site of damage during a freeze-thaw cycle (see Steponkus et al., 1993). Additionally, we wanted to determine whether the proteins, which are hydrophilic, have a particularly strong avidity for water. A stimulus for this was the suggestion by Volger and Heber (1975) that extensive water binding by the putative cryoprotective proteins of cabbage and spinach might provide a protective environment in the proximity of the membranes during freezing and result in membrane stabilization.

It was anticipated that the studies described in the accompanying papers (Uemura et al., 1996; Webb et al., 1996) would require production of milligram amounts of highly purified COR15am and COR6.6. Pilot experiments suggested that this would be problematic using Arabidopsis plants as the protein source and, thus, we chose to express the polypeptides at high levels in *E. coli* (Fig. 2). Standard biochemical methods were then used to purify the recombinant proteins to near homogeneity (Fig. 3). Analyses of the purified COR15am^r polypeptide indicate that its amino acid sequence should be identical to that of the COR15am found in Arabidopsis chloroplasts, including the lack of an N-terminal Met residue. There was no indication that expression of the polypeptide in *E. coli* resulted in production of a denatured protein because it remained soluble (did not form inclusion bodies) and co-migrated on nondenaturing polyacrylamide gels with the major species of COR15am found in chloroplasts. COR15am^r also co-migrated with the major species of chloroplast COR15am on two-dimensional O'Farrell gels. Taken together, these data indicate that the proposed site of COR15a processing (Lin and Thomashow, 1992) is correct and that there is no difference in charge or quaternary structure between COR15am^r and the major species of COR15am in plants. The data also suggest that COR15am does not form a stable multimeric protein with other, unrelated polypeptides. However, the migration of COR15am^r and COR15am on nondenaturing polyacrylamide gels raises the possibility that the polypeptides form a multimeric complex. If so, the multimer in plants could be either a homomeric complex consisting of COR15am polypeptides or a heteromeric complex consisting of COR15am and a closely related polypeptide(s). Indeed, immediately downstream of the *COR15a* gene is *COR15b*, an apparent homolog of *COR15a* (Wilhelm and Thomashow, 1993). The COR15b polypeptide is predicted to be

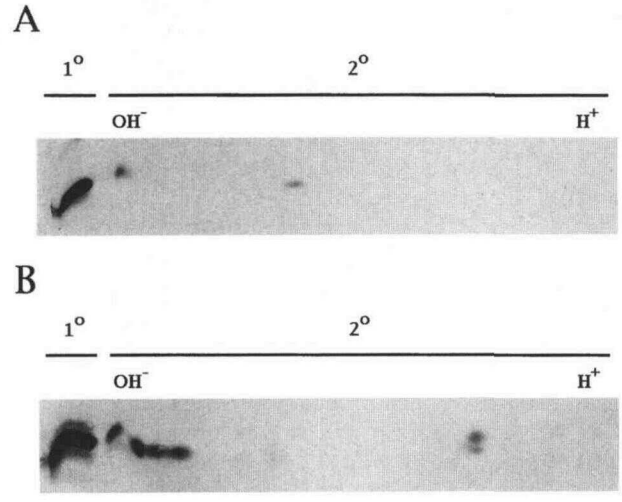

Figure 7. Migration of COR6.6^r and COR6.6 on two-dimensional O'Farrell gels. A, Purified sample of COR6.6^r (300 ng). B, Total soluble proteins from plants cold acclimated for 6 weeks (100 μg). Protein samples were prepared and fractionated on two-dimensional O'Farrell gels, and COR6.6^r/COR6.6 were detected by immunoblot analysis as described in "Materials and Methods." The region of the gel corresponding to the second dimension (i.e. the region containing proteins that had been first separated in an IEF gel) is indicated as 2°. The pH gradient ranged from approximately pH 6.5 to 8.2. In addition, samples of COR6.6^r (300 ng) and plant protein (100 μg) that were fractionated in only the second dimension (SDS-PAGE) are indicated as 1°. A comparison of the 1° and 2° portions of the gel give an estimate of the amount of protein sample that entered the IEF gel.

targeted to chloroplasts and to be processed to a mature polypeptide, COR15bm, which is very similar to COR15am in amino acid sequence, molecular mass, and pI.

Whereas COR15am^r appears to be identical to the major species of COR15am, COR6.6^r and COR6.6 have certain differences. One resulted from the cloning procedure, which necessitated changing the second amino acid of COR6.6 from a Ser to an Ala. Another is that the N-terminal Met is removed from the recombinant polypeptide in *E. coli*; the COR6.6 polypeptide in plants presumably retains this amino acid. Potentially the most important difference, however, regards the apparent modification of COR6.6 in vivo. In particular, the data suggest that a significant portion (perhaps half) of the COR6.6 population in plants is more acidic than COR6.6^r. A likely explanation for this alteration in pI is that the polypeptide becomes phosphorylated in vivo. Given that phosphorylation is a common mechanism used to either enhance or reduce the activity of proteins, it is possible that the level of COR6.6^r activity differs from that of much of the COR6.6 found in plants.

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