

# DNA Sequence Analysis of a Complementary DNA for Cold-Regulated *Arabidopsis* Gene *cor15* and Characterization of the COR15 Polypeptide<sup>1</sup>

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## ABSTRACT

Previous studies have indicated that changes in gene expression occur in *Arabidopsis thaliana* L. (Heyn) during cold acclimation and that certain of the *cor* (cold-regulated) genes encode polypeptides that share the unusual property of remaining soluble upon boiling in aqueous solution. Here, we identify a cDNA clone for a cold-regulated gene encoding one of the “boiling-stable” polypeptides, COR15. DNA sequence analysis indicated that the gene, designated *cor15*, encodes a 14.7-kilodalton hydrophilic polypeptide having an N-terminal amino acid sequence that closely resembles transit peptides that target proteins to the stromal compartment of chloroplasts. Immunological studies indicated that COR15 is processed *in vivo* and that the mature polypeptide, COR15m, is present in the soluble fraction of chloroplasts. Possible functions of COR15m are discussed.

In many species of higher plants, a period of exposure to low nonfreezing temperatures results in an increased level of freezing tolerance (17). Considerable effort has been directed at understanding the molecular basis of this cold acclimation response. Comparative biochemical analyses have revealed a variety of changes that occur during cold acclimation, including alterations in lipid, protein, and carbohydrate composition (10, 25, 26). In most cases, however, the role that a given change has in the cold acclimation process remains poorly understood.

In 1970, Weiser (30) suggested that cold acclimation might involve changes in gene expression. Since then, it has been established that changes in gene expression occur during cold acclimation in a wide range of plant species (10, 26). It is not known, however, whether cold-regulated genes have critical roles in freezing tolerance. To address this issue, investigators have begun to isolate and characterize genes that are induced during cold acclimation. Although no functions have yet been determined for these genes, results have been intriguing. Mohapatra *et al.* (19), for example, found that the levels of expression of three cold-regulated genes from alfalfa correlate positively with the freezing tolerances of four different alfalfa

cultivars. Kurkela and Franck (14) reported that a cold-regulated gene from *Arabidopsis thaliana*, *kin1*, encodes an alanine-rich polypeptide that has amino acid sequence similarities with certain fish antifreeze proteins. Whether KIN1 has antifreeze properties *in vitro* or *in vivo* is not known.

We have found that certain *cor*<sup>2</sup> genes of *Arabidopsis* and wheat encode polypeptides that share the unusual property of remaining soluble upon boiling in aqueous solution (18). One of the cold-regulated “boiling-stable” polypeptides of *Arabidopsis*, COR47, is related to certain LEA (late embryogenesis abundant) proteins (8), a group of hydrophilic polypeptides (also boiling-stable) that have been hypothesized to have roles in water stress tolerance (1, 3, 13). The possible relevance of this to cold acclimation is that the cellular damage that results from a freeze-thaw cycle is due in large part to the dehydration that occurs during freezing (17, 25). These and other considerations have led us to speculate that COR47 might act as a cryoprotectant by helping plant cells withstand the dehydration stress associated with freezing (18).

Here, we continue to characterize *Arabidopsis cor* genes and the polypeptides that they encode. Specifically, we have identified a cDNA clone for the *cor* gene that encodes the 15-kD boiling-stable polypeptide, COR15. DNA sequence analysis indicates that the COR15 polypeptide is hydrophilic and that it has an N-terminal amino acid sequence that closely resembles transit peptides that target proteins to the stromal compartment of chloroplasts. Immunological studies indicate that COR15 is processed *in vivo* and that the mature polypeptide is present in chloroplasts. Possible roles for COR15 in cold acclimation are discussed.

## MATERIALS AND METHODS

### Plant Material

*Arabidopsis thaliana* L. (Heyn) ecotype Landsberg *erecta* was grown in controlled environment chambers at 21°C under constant light as previously described (9, 18). Plants were cold acclimated by transferring them to controlled environment chambers at 4°C (constant light) for 3 or more days.

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<sup>2</sup> Abbreviations: *cor* (cold-regulated); poly(A<sup>+</sup>) RNA, polyadenylated RNA.

### cDNA Clones

pLCT10A and pLCT10B are homologous to a previously described *cor* cDNA clone, pHH67 (11). Both pLCT10A and pLCT10B have the same 709-base pair *EcoRI* insert cloned in the *EcoRI* site of pBluescript SK<sup>-</sup> (Stratagene, La Jolla, CA) but in opposite orientations. pLCT10A was isolated by screening plaque lifts (20) of a cDNA library for recombinant phage that hybridized with the insert from pHH67; probes were prepared by nick translation or random priming (20). The cDNA library screened was prepared from poly(A<sup>+</sup>) RNA isolated from *A. thaliana* (Columbia) seedlings that had been grown at 22°C for approximately 15 d and cold acclimated at 4°C for 3 d. The cDNA library was constructed in λZAP (Stratagene) as previously described (11). Recombinants carrying inserts related to pHH67 were plaque purified, and the inserts were "subcloned" into pBluescript SK<sup>-</sup> by biological rescue as recommended by the supplier (Stratagene). One of the clones chosen for further study was pLCT10A. The orientation of the *EcoRI* insert in pLCT10A was reversed using standard recombinant DNA methods (20) to give pLCT10B. DNA sequence analysis indicated that the insert of pLCT10A and pLCT10B represented the same gene as pHH67 but had an additional sequence from the 5' end of the transcript.

### DNA Sequencing

DNA sequences were determined by the dideoxy method of Sanger *et al.* (21) using Sequenase (U.S. Biochemical, Cleveland, OH) according to the manufacturer's instructions. Deletions of the insert were generated using exonuclease III. Single- and double-stranded templates were prepared as previously described (8, 18). The complete sequence of each strand of the insert was determined. Sequence analysis was done using the PROsis and DNAsis programs of Hitachi (San Bruno, CA) and the CGC programs (version 6.0) of the University of Wisconsin Biotechnology Center (Madison, WI).

### In Vitro Transcription/Translation Reactions

pLCT10A and pLCT10B were linearized by digestion with *Bam*HI and the inserts transcribed *in vitro* with T7 RNA polymerase (Promega, Madison, WI) using the T7 promoter carried on the pBluescript vector. The resulting transcripts were extracted with phenol:chloroform (1:1), precipitated with ethanol, and in certain cases "capped" *in vitro* using an mCAP mRNA-capping kit (Stratagene). Transcription products were translated *in vitro* using the rabbit reticulocyte lysate system (Promega) containing [<sup>35</sup>S]methionine. Boiling-stable polypeptides were prepared as described previously (18). Radioactive polypeptides were fractionated by SDS-PAGE (16) on 15% (w/v) polyacrylamide gels and visualized by autoradiography (9).

### Hybrid Arrest/In Vitro Translation Reactions

Poly(A<sup>+</sup>) RNA isolated from leaves of cold-acclimated plants (9, 11) was hybridized with single-stranded DNA prepared from pLCT10A and pBluescript SK<sup>-</sup> as previously described (18), and the "arrest" reactions were translated *in vitro* as described above.

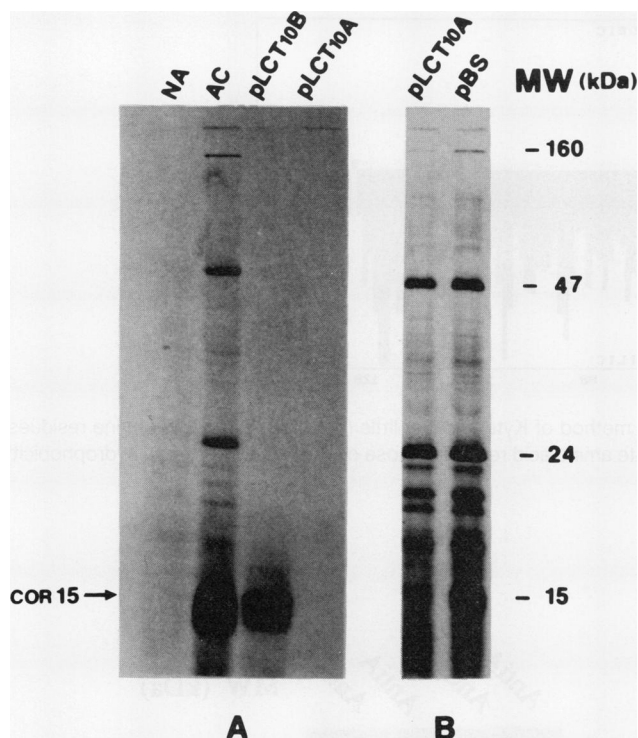
### Antiserum

Antiserum that recognized COR15 was raised by immunizing rabbits with a protein A-COR15 fusion protein. A gene encoding the hybrid protein was created by ligating the *EcoRI* cDNA insert of pLCT10B into the *EcoRI* site of pRIT2T, a protein A fusion vector (Pharmacia, Uppsala, Sweden). The recombinant plasmid was transformed into *Escherichia coli* N4830-1 (Pharmacia) and the fusion protein expressed as recommended by the supplier. Cells were disrupted using a French Press (Aminco, Urbana, IL) at 16,000 psi, and the extract was centrifuged at 10,000g for 15 min. The supernatant was collected, and the protein A-COR15 fusion was enriched by affinity chromatography using a column of immunoglobulin G Sepharose (Pharmacia) according to manufacturer's instructions. The fusion protein was further purified by preparative SDS-PAGE. Gel slices containing the 41-kD fusion protein were homogenized in a buffer consisting of 0.1 M Tris-HCl (pH 8.0), 0.1% (w/v) SDS using a mortar and pestle, and the suspension was stirred at room temperature for 5 h. The material was centrifuged at 10,000g for 15 min, and the fusion protein in the supernatant was concentrated using a Centricon 10 filter (Amicon, Beverly, MA). Analogous procedures utilizing the unmodified pRIT2T vector were used to obtain the protein A polypeptide. New Zealand white rabbits were immunized with the protein preparations by subcutaneous injection of 80 μg of protein and boosted once after 4 weeks with the same amount of protein.

Antiserum recognizing COR160, an *Arabidopsis* cold-regulated boiling-stable polypeptide (9, 18), was obtained from Sarah Gilmour (S. Gilmour and M. Thomashow, unpublished). Antisera to carbonic anhydrase (5) and glycolate oxidase (29) of spinach were kindly provided by Chris Somerville.

### Chloroplast Isolation

Chloroplasts were isolated by modification (S. Hugly and C. Somerville, unpublished data) of a previously described procedure (24). Leaves (10 g) harvested from 2- to 3-week-old plants (rosette stage) were immersed in ice water for 5 min, blotted dry, placed in 150 mL of cold grinding buffer (20 mM Tris-HCl [pH 8.4], 1% [w/v] BSA, 1.25% [w/v] Ficoll-400, 2.5% [w/v] Dextran-40, 0.45 M sorbitol, 10 mM EDTA, 1 mM DTT), cut into small pieces with scissors, and ground for 10 s in a Tissumizer (Tekmar, Cincinnati, OH) at maximum speed. The homogenates were passed through Miracloth (Calbiochem, San Diego, CA) and centrifuged at 1400g for 1 min. Pelleted material was gently resuspended with a camel hair paint brush in 2 mL resuspension buffer (100 mM Tris [pH 7.9], 300 mM glycerol, 1 mM MgCl<sub>2</sub>, 1 mM DTT), layered onto a discontinuous Percoll gradient (1 mL of 60% [v/v] Percoll, 10 mL of 25% [v/v] Percoll in resuspension buffer), and centrifuged in a swinging bucket rotor (Sorval HB-4) at 6000 rpm for 3 min. Chloroplasts banding at the interface between the Percoll layers were collected, suspended in 5× volume of resuspension buffer, and collected by centrifugation at 1300g for 1 min.



**Figure 1.** Evidence that the cDNA insert in pLCT10A and pLCT10B corresponds to the transcript encoding COR15. A, pLCT10A and pLCT10B were transcribed *in vitro* using T7 polymerase, the resulting transcripts were translated *in vitro*, and the boiling-stable polypeptide fraction was analyzed by SDS-PAGE (18). Lanes NA and AC contain the boiling-stable polypeptide fractions synthesized by *in vitro* translation of poly(A<sup>+</sup>) RNA isolated from nonacclimated and cold-acclimated plants, respectively. B, Poly(A<sup>+</sup>) RNA isolated from cold-acclimated plants was hybridized with single-stranded DNA prepared from either pLCT10A or the cloning vector (pBS) and translated *in vitro*, and the boiling-stable proteins synthesized were analyzed by SDS-PAGE on 15% (w/v) gels. The data presented are autoradiographs of the SDS-PAGE gels.

### Protein Preparations

Total soluble protein was prepared by grinding plant tissue in liquid N<sub>2</sub> followed by grinding in extraction buffer (50 mM Tris [pH 8.0], 5 mM EDTA, 2.5% [w/v] PVP) (4 mL buffer/g tissue). The material was then centrifuged (10,000g, 10 min), the pellet was discarded, and the proteins in the supernatant were collected by addition of 3 volumes of acetone followed by centrifugation (14,000g for 10 min).

Chloroplast proteins were separated into soluble and membrane fractions by suspending isolated chloroplasts in 5× volume of lysis buffer (100 mM Tris [pH 7.9, 5 mM EDTA, 1 mM PMSF), freezing the suspension at -20°C for 30 min, thawing it at 37°C for 5 min, mixing it using a Vortex (about 1 min), and centrifuging it in a microcentrifuge at approximately 14,000g for 15 min. Proteins in the supernatant and pellet were collected by adding 2 volumes of acetone followed by centrifugation (14,000g for 10 min) and designated the soluble and membrane fractions, respectively.

### Immunoblot Analysis

Proteins were fractionated by SDS-PAGE and transferred to nitrocellulose (Schleicher and Schuell, Keene, NH) as described before (27). Immunoblots were then treated with antisera, and bound antibody was detected using protein A-conjugated alkaline phosphatase (Sigma) as described previously (2).

## RESULTS

### Identification of a cDNA Encoding COR15

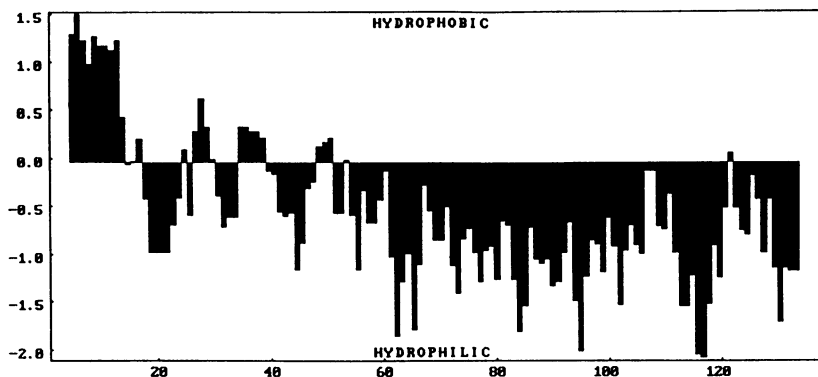
We recently isolated cDNA clones for four *Arabidopsis cor* genes (11). Preliminary hybrid-select *in vitro* translation experiments indicated that one of these clones, pHH67, encoded a 15-kD boiling-stable polypeptide designated COR15 (not shown). Further experiments with pLCT10A and pLCT10B, cDNA clones homologous to pHH67 (see "Materials and Methods"), confirmed these results. Hybrid-arrest *in vitro* translation experiments indicated that single-stranded DNA prepared from pLCT10A hybridized with the transcript encoding the boiling-stable COR15 polypeptide, whereas single-stranded DNA prepared from the pBluescript vector did not (Fig. 1B). Furthermore, when pLCT10B was transcribed *in vitro* using T7 polymerase (the vector carries a T7 promoter) and translated *in vitro*, a boiling-stable polypeptide of 15 kD was synthesized (Fig. 1A). This polypeptide was not synthesized in *in vitro* transcription/translation reactions using pLCT10A, which has the same insert as pCTL10B but in opposite orientation.

### DNA Sequence Analysis

The DNA sequence of the cDNA insert cloned in pLCT10A and pLCT10B was determined (Fig. 2). The data indicate that

1	AAA AAC TCC TCC TTT CAT TTC CAA ACA AAA ACT TCT TTT TAT TCT CAC	48
49	ATC TTA AAG ATC TCT CTC ATG GCG ATG TCT TTC TCA GGA GCT GTT CTC	96
	Met Ala Met Ser Phe Ser Gly Ala Val Leu	10
97	ACT GGT ATG GCT TCT TCT TTC CAC AGC GGA GCC AAG CAG AGC AGC TTC	144
11	Thr Gly Met Ala Ser Ser Phe His Ser Gly Ala Lys Gln Ser Ser Phe	26
145	GGC GCT GTC AGA GTC GGC CAG AAA ACT CAG TTC GTC GTC GTT TCT CAA	192
27	Gly Ala Val Arg Val Gly Gln Lys Thr Gln Phe Val Val Val Ser Gln	42
193	CGC AAG AAG TCG TTG ATC TAC GCC GCT AAA GGT GAC GGC AAC ATC CTC	240
43	Arg Lys Lys Ser Leu <u>Ile Tyr Ala*Ala</u> Lys Gly Asp Gly Asn Ile Leu	58
241	GAT GAC CTC AAC GAG GCC ACA AAG AAA GCT TCA GAT TTC GTG ACG GAT	288
59	Asp Asp Leu Asn Glu Ala Thr Lys Lys Ala Ser Asp Phe Val Thr Asp	74
289	AAA ACA AAA GAG GCA TTA GCA GAT GGT GAG AAA GCG AAA GAC TAC GTT	336
75	Lys Thr Lys Glu Ala Leu Ala Asp Gly Glu Lys Ala Lys Asp Tyr Val	90
337	GTT GAA AAA AAC AGT GAA ACC GCA GAT ACA TTG GGT AAA GAA GCT GAG	384
91	Val Glu Lys Asn Ser Glu Thr Ala Asp Thr Leu Gly Lys Glu Ala Glu	106
385	AAA GCT GCG GCG TAT GTG GAG GAG AAA GGA AAA GAA GCC GCA AAC AAG	432
107	Lys Ala Ala Ala Tyr Val Glu Glu Lys Gly Lys Glu Ala Ala Asn Lys	122
433	GCG GCA GAG TTC GCG GAG GGT AAA GCA GGA GAG GCT AAG GAT GCC ACA	480
123	Ala Ala Glu Phe Ala Glu Gly Lys Ala Gly Glu Ala Lys Asp Ala Thr	138
481	AAG TAG GGT CTT ACC TAA TCA GTT AAT TTC AAG CAC TTA AAC TCG TAG	528
139	Lys ***	
529	ATA TAT TGA TCC ATA TCC TCT CTC TTC ATG TTT AAT AGT ACT TAC AAT	576
577	AAG ATG AGT CTG TTG TAA TTT CTA TTA ATT TCA CAT CAC AAC TGA AAT	624
625	AAG ATA TGG TAT CCA CAG TCA CCG TCA CAT TCT TTA ATT TTG TGC AAA	672
673	ATA TTC AAT AGA CAA ATT AAA TAA AAA AAA AAA	

**Figure 2.** Nucleic acid sequence of the cDNA insert in pLCT10A/pLCT10B and the deduced amino acid sequence of COR15. The loosely defined consensus cleavage site for chloroplast transit peptides (7) is underlined, and the predicted site of cleavage is indicated by an asterisk (see text).



**Figure 3.** Hydropathy profile of COR15. Plots were made according to the method of Kyte and Doolittle (15) using a window of nine residues. Negative values indicate hydrophilic regions. Numbers on the abscissa indicate amino acid residues; those on the ordinate indicate hydrophobicity values.

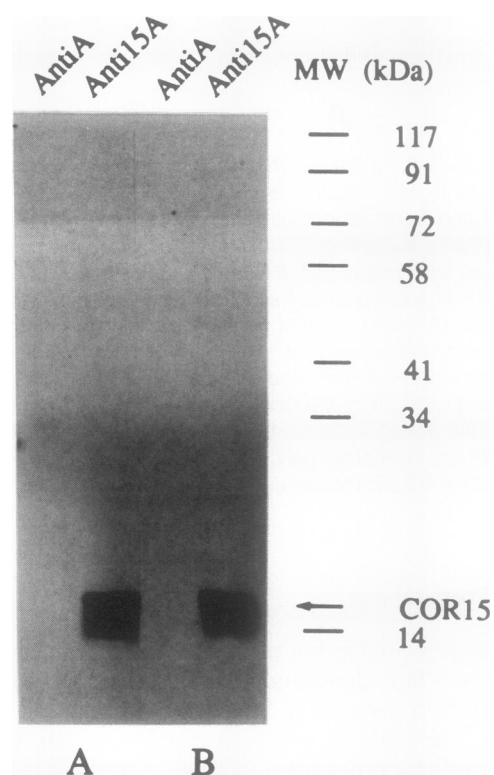
the insert has an open reading frame that could encode a 139-amino acid polypeptide with a predicted molecular mass of 14,604 D, a value consistent with the *in vitro* transcription/translation experiment presented above (Fig. 1). The gene encoding this polypeptide was designated *cor15*. The deduced polypeptide, designated COR15, had a high alanine (17.9 mol%) and lysine (14.3 mol%) content and was devoid of cysteine, tryptophan, and proline residues. The hydropathy profile of the polypeptide indicated that the N-terminal third of COR15 had both hydrophobic and hydrophilic regions but that the latter two-thirds of the polypeptide was primarily hydrophilic (Fig. 3). Analysis of the potential secondary structure of the polypeptide using the algorithm of Robson (6) indicated that the latter two-thirds of the polypeptide (from approximately residue 50 to the end) was likely to assume an  $\alpha$ -helical configuration (not shown). Furthermore, a helical wheel diagram (22) indicated that the helix would be amphipathic (not shown). Computer searches of the Genbank (release 68, June 1991), EMBL (release 27, May 1991), SwissProt (release 18.0, May 1991), and PIR Prot (release 28.0, May 1991) data bases did not reveal extensive nucleic acid or amino acid sequence homology between the *cor15* transcript or polypeptide and any previously sequenced genes or polypeptides.

#### Preparation of Antiserum to COR15

Antiserum was raised to a fusion protein consisting of protein A and COR15 (see "Materials and Methods"). Two lines of evidence indicated that the antiserum recognized COR15: it precipitated a protein of 15 kD from *in vitro* translations of RNA isolated from cold-acclimated *Arabidopsis* (Fig. 4A), and it precipitated the COR15 polypeptide prepared by *in vitro* transcription/translation of pLCT10B (Fig. 4B). Antisera prepared against protein A alone did not precipitate the 15-kD polypeptide in either sample (Fig. 4).

#### Processing of COR15 *in Vivo*

The antiserum raised to COR15 was used to detect the presence of COR15 in plant tissues. Total soluble protein was prepared from cold-acclimated and nonacclimated plants,



**Figure 4.** Antibody prepared against the protein A-COR15 fusion polypeptides recognizes COR15. A, Poly(A<sup>+</sup>) RNA isolated from cold-acclimated plants was translated *in vitro*, the polypeptide products were immunoprecipitated with either the antiserum raised against protein A (AntiA) or the antiserum raised against the protein A-COR15 fusion (Anti15A), and the precipitated polypeptides were fractionated by SDS-PAGE on 15% (w/v) gels. B, pLCT10B was transcribed *in vitro* using T7 polymerase, the transcripts were translated *in vitro*, and the polypeptides synthesized were immunoprecipitated and analyzed as in A. The data presented are autoradiographs of the resulting SDS-PAGE gels.

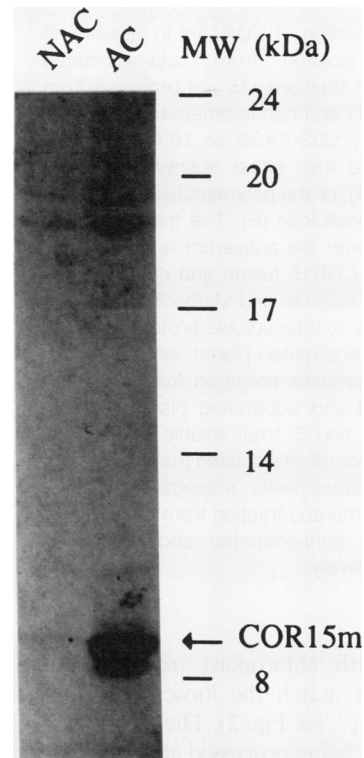
fractionated by ammonium sulfate precipitation, and subjected to SDS-PAGE. Immunoblots were then prepared and treated with the COR15 antiserum. The data indicated that COR15 was present in the 40 to 60% ammonium sulfate cuts of the protein samples from cold-acclimated plants but not in the analogous samples from nonacclimated plants (Fig. 5). The apparent molecular mass of the COR15 polypeptide, however, was only about 9 kD, considerably smaller than the 15-kD *in vitro* translation product (see Fig. 1). These data indicated that the COR15 polypeptide might be processed *in vivo*; the putative mature peptide was designated COR15m.

#### Similarities between the N-Terminal End of COR15 and Chloroplast Transit Peptides

Inspection of the COR15 amino acid sequence indicated that the N-terminal third of the polypeptide had several features in common with transit peptides that target proteins to the stromal compartment of chloroplasts (7). First, the sequence of COR15 at residues 48 to 51 (Fig. 2), Ile-Tyr-Ala-Ala, matched the loosely defined consensus cleavage site for chloroplast transit peptides, (Val/Ile)-X-(Ala/Cys)-Ala (proteolytic cleavage occurs between the terminal Ala residue and the penultimate Ala/Cys residue). Cleavage of COR15 at this processing site would result in a polypeptide with a deduced molecular mass of 9.4 kD, a size that was consistent with the COR15 product observed *in vivo* (Fig. 5). Second, chloroplast transit peptides typically have one or more arginine residues in positions -6 to -10 relative to the putative cleavage site; COR15 had an arginine residue at position -8 (Fig. 2). Third, chloroplast transit peptides generally have a high content of serine and threonine residues and low numbers of acidic amino acid residues. Accordingly, the putative COR15 transit peptide had a serine plus threonine content of 22% and contained no glutamic or aspartic acid residues (Fig. 2). Finally, three structural domains can be discerned in chloroplast transit peptides: an uncharged amino-terminal domain, a central positively charged domain lacking acidic residues, and a carboxy-terminal domain with a high potential for forming an amphiphilic  $\beta$ -strand. In agreement, COR15 residues 1 to 18 were without charge (Fig. 2), residues 18 to 35 had four positive charges and no acidic residues (Fig. 2), and residues 27 to 41 were predicted (6) to form a  $\beta$ -sheet (not shown).

#### Detection of COR15m in Chloroplast Preparations

Initial experiments indicated that COR15m was present in total soluble protein extracts prepared from leaves of cold-acclimated plants (Fig. 6, lanes 3), but, as might be expected for a chloroplast protein, it was not detected in total soluble protein extracts prepared from the roots of these plants (Fig. 6, lanes 1). Chloroplasts were then purified from the leaves of cold-acclimated plants, total soluble proteins were prepared, and, indeed, COR15m was detected in the extracts (Fig. 6, lanes 4). Similarly, carbonic anhydrase, a stromal chloroplast protein, was detected in protein extracts prepared from whole leaves and chloroplasts (Fig. 7B). In contrast, COR160 (Fig. 7A), another cold-regulated boiling-stable polypeptide of *Arabidopsis* (9, 18), and glycolate oxidase (not shown), a per-



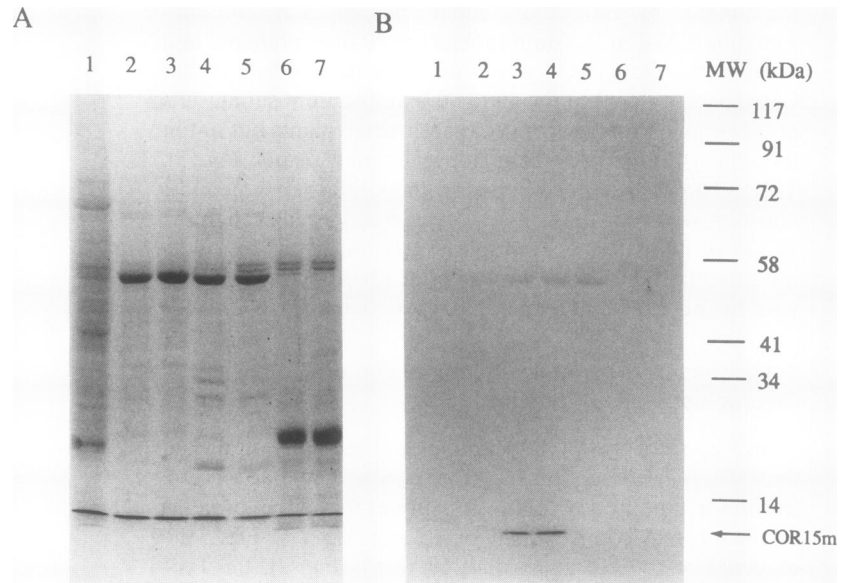
**Figure 5.** Evidence that the COR15 polypeptide is processed *in vivo*. Total soluble protein (15  $\mu$ g) prepared from leaves of nonacclimated (NAC) and cold-acclimated (AC) plants was fractionated by ammonium sulfate precipitation, and the polypeptides in the 40 to 60% cut were separated by SDS-PAGE on an 11 to 18% (w/v) gradient gel (32 cm long). The bottom half of the gel was cut to fit a conventional electroblot apparatus, and the proteins were transferred to nitrocellulose. The immunoblots were then treated with the protein A-COR15 antiserum, and bound antibody was detected as described in "Materials and Methods."

oxisomal enzyme, were present in soluble protein extracts prepared from total leaf tissue but were not detected in extracts prepared from the purified chloroplasts. COR15m was not detected in soluble protein extracts prepared from leaves (Fig. 6, lanes 2) or chloroplasts (Fig. 6, lanes 5) isolated from nonacclimated plants or in membrane fractions prepared from chloroplasts isolated from either cold-acclimated (Fig. 6, lanes 7) or nonacclimated plants (Fig. 6, lanes 6).

#### DISCUSSION

The results of DNA sequence and immunoblot analyses indicate that the *cor15* gene of *Arabidopsis* encodes a 14.7-kD cold-regulated polypeptide, COR15, that is processed *in vivo* to a polypeptide of about 9 kD. The mature protein, designated COR15m, is soluble, hydrophilic, and predicted to form an amphipathic  $\alpha$ -helix. Furthermore, COR15m appears to be located in chloroplasts. In particular, COR15m can be detected in soluble protein extracts prepared from chloroplasts purified on Percoll gradients (Fig. 6). In addition, amino acid sequence comparisons indicate that the N-terminal sequence of COR15 has a number of characteristics in

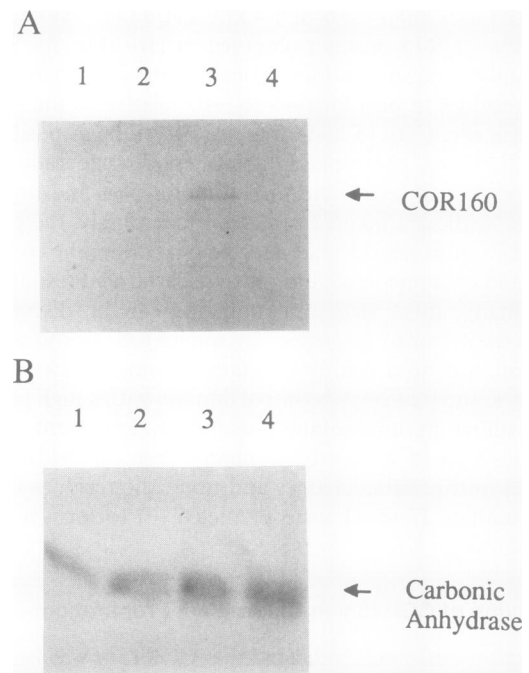
**Figure 6.** Detection of COR15m in leaves and chloroplasts isolated from cold-acclimated plants. Protein fractions (15  $\mu$ g) prepared from cold-acclimated and nonacclimated plants were fractionated by SDS-PAGE on 10% (w/v) gels. The gels were then either stained with Coomassie blue (A), or the polypeptides were transferred to nitrocellulose (B). The transfers were then treated with the antiserum raised against the protein A-COR15 fusion and developed as described in "Materials and Methods." The samples are: lanes 1, total soluble protein prepared from roots of acclimated plants; lanes 2 and 3, total soluble proteins prepared from leaves of nonacclimated and acclimated plants, respectively; lanes 4 and 5, total soluble protein prepared from chloroplasts isolated from acclimated and nonacclimated plants, respectively; lanes 6 and 7, the membrane fraction from chloroplasts isolated from nonacclimated and acclimated plants, respectively.



common with chloroplast transit peptides, including sequences that match the loose consensus stromal targeting cleavage site (7; see Fig. 2). The size of COR15m is consistent with COR15 being processed at the putative consensus cleavage site; the apparent molecular mass of COR15m is about 9 kD (Fig. 5), whereas the predicted molecular mass of COR15m, assuming processing at residue 50 of COR15 (the putative cleavage site) is 9.4 kD (Fig. 2).

Attempts to determine directly the N-terminal sequence of COR15m, and thus confirm the site of processing, have failed, apparently due to chemical blockage of the N-terminal amino acid (C. Lin, unpublished data). However, we have constructed a gene that contains the COR15m sequence fused to a methionine codon, have expressed the polypeptide in *E. coli*, and have found that it comigrates with authentic COR15m (prepared from chloroplasts) on SDS-PAGE (C. Lin, unpublished results). Taken together, these data indicate that at least some of the cellular COR15m is located in chloroplasts. Confirmation of this conclusion and a determination of the suborganelle location of COR15m will be accomplished by future immunolocalization studies. Finally, it should be noted that our data do not rule out the possibility that COR15 is targeted to more than one cellular compartment.

A major challenge now is to determine the function(s) of COR15m. One intriguing possibility is suggested by the work of Heber and colleagues (12, 28). These investigators have found that cold-acclimated spinach and cabbage, but not nonacclimated plants, synthesize proteins that can protect isolated thylakoid membranes against freeze damage *in vitro*. The cryoprotective activity of these proteins is high; on a molar basis, they are >10,000 times more effective than sucrose in protecting thylakoids against damage caused by a freeze-thaw cycle. To date, none of these cryoprotective proteins have been isolated to purity. However, initial studies (12, 28) indicate that they have a number of properties in common with COR15m: they are cold regulated and hydrophilic, they remain soluble upon boiling in aqueous solution,



**Figure 7.** Detection of COR160 and carbonic anhydrase in protein fractions isolated from leaves of nonacclimated and cold-acclimated plants. Immunoblots were prepared from protein preparations (15  $\mu$ g) fractionated by SDS-PAGE on 10% (w/v) gels. The transfers were then treated with antisera to either COR160 (A) or carbonic anhydrase (B) and developed as described in "Materials and Methods." The samples are: lanes 1 and 2, total protein prepared from chloroplasts isolated from nonacclimated and acclimated plants, respectively; lanes 3 and 4, total soluble protein prepared from leaves of acclimated and nonacclimated plants, respectively.

they are small (about 10–30 kD), and at least some of them appear to be present in chloroplasts (see discussion of ref. 12). The obvious question raised is whether COR15m also has cryoprotective properties. Current efforts are directed at determining whether this is the case. The key question, of course, is whether COR15m and the spinach and cabbage proteins have cryoprotective roles *in vivo*. In particular, do they have specific functions in protecting chloroplasts against freeze-induced damage? Ongoing experiments, including the construction of transgenic *Arabidopsis* that either overexpress or underexpress *cor15*, will hopefully provide an answer to this question.

Previous studies have indicated that *cor15* is expressed in response to low temperature (11), a result that is consistent with COR15m having a role in freezing tolerance. However, *cor15* transcripts also accumulate in response to water stress (11). Indeed, each of the four *cor* genes for which we have isolated cDNA clones is induced in response to water deprivation (11). Is there a rationale for why genes involved in cryoprotection might be activated under drought conditions? One possibility relates to the fact that the damage plant cells suffer in response to a freeze-thaw cycle results largely from the cellular dehydration that occurs during freezing (17, 25). Thus, tolerance to freezing must include tolerance to water stress. It therefore seems reasonable to speculate that freezing and drought tolerance might involve related mechanisms and that such mechanisms might include the activation of related or identical genes. Indeed, water stress has been shown to increase the freezing tolerance of certain cereal (23) and *Brassica* (4) species. Future experiments will be directed at determining whether *cor15* and the other drought-regulated *Arabidopsis cor* genes are examples of such hypothetical "dual-action" environmental stress genes.

#### ACKNOWLEDGMENTS

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#### LITERATURE CITED

- Baker J, Steele C, Dure L III (1988) Sequence and characterization of 6 *Lea* proteins and their genes from cotton. *Plant Mol Biol* **11**: 277–291
- Blake MS, Johnston KH, Russell-Jones GT, Gotschlich EC (1984) A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on western blots. *Anal Biochem* **136**: 175–179
- Close TJ, Kortt AA, Chandler PM (1989) A cDNA-based comparison of dehydration-induced proteins (dehydrins) in barley and corn. *Plant Mol Biol* **13**: 95–108
- Cox W, Levitt J (1976) Interrelation between environmental factors and freezing resistance of cabbage leaves. *Plant Physiol* **57**: 553–555
- Fawcett TW, Browse JA, Volokita M, Bartlett SG (1990) Spinach carbonic anhydrase primary structure deduced from the sequence of a cDNA clone. *J Biol Chem* **265**: 5414–5417
- Garnier J, Osguthorpe DJ, Robson B (1978) Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J Mol Biol* **120**: 97–120
- Gavel Y, von Heijne G (1990) A conserved cleavage-site motif in chloroplast transit peptides. *FEBS Lett* **261**: 455–458
- Gilmour SJ, Artus NN, Thomashow MF (1992) cDNA sequence analysis and expression of two cold-regulated genes of *Arabidopsis thaliana*. *Plant Mol Biol* **18**: 13–21
- Gilmour SJ, Hajela RK, Thomashow MF (1988) Cold acclimation in *Arabidopsis thaliana*. *Plant Physiol* **87**: 745–750
- Guy CL (1990) Cold acclimation and freezing stress tolerance: role of protein metabolism. *Annu Rev Plant Physiol Plant Mol Biol* **41**: 187–223
- Hajela RK, Horvath DP, Gilmour SJ, Thomashow MF (1990) Molecular cloning and expression of *cor* (cold-regulated) genes in *Arabidopsis thaliana*. *Plant Physiol* **93**: 1246–1252
- Hincha DK, Heber U, Schmitt JM (1989) Freezing ruptures thylakoid membranes in leaves, and rupture can be prevented *in vitro* by cryoprotective proteins. *Plant Physiol Biochem* **27**: 795–801
- Jacobsen JV, Shaw DC (1989) Heat-stable proteins and abscisic acid action in barley aleurone cells. *Plant Physiol* **91**: 1520–1526
- Kurkela S, Franck M (1990) Cloning and characterization of a cold- and ABA-inducible *Arabidopsis* gene. *Plant Mol Biol* **15**: 137–144
- Kyte J, Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. *J Mol Biol* **157**: 105–132
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685
- Levitt J (1980) Responses of Plants to Environmental Stress. Chilling, Freezing, and High Temperature Stresses, Ed 2. Academic Press, New York
- Lin C, Guo WW, Everson E, Thomashow MF (1990) Cold acclimation in *Arabidopsis* and wheat. A response associated with expression of related genes encoding 'boiling-stable' polypeptides. *Plant Physiol* **94**: 1078–1083
- Mohapatra SS, Wolfrum L, Poole RJ, Dhindsa RS (1989) Molecular cloning and relation to freezing tolerance of cold-acclimation-specific genes of alfalfa. *Plant Physiol* **89**: 375–380
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning. A Laboratory Manual*, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**: 5463–5467
- Schiffer M, Edmundson AB (1967) Use of helical wheels to represent the structure of proteins and identify segments with helical potentials. *Biophys J* **7**: 121–135
- Siminovitch D, Cloutier Y (1983) Drought and freezing tolerance and adaptation in plants: some evidence of near equivalences. *Cryobiology* **20**: 487–503
- Somerville CR, Somerville SC, Ogren WL (1981) Isolation of photosynthetically active protoplasts and chloroplasts from *Arabidopsis thaliana*. *Plant Sci Lett* **21**: 89–96
- Steponkus PL, Lynch DV (1989) Freeze/thaw-induced destabilization of the plasma membrane and the effects of cold acclimation. *J Bioenerg Biomembr* **21**: 21–41
- Thomashow MF (1990) Molecular genetics of cold acclimation in higher plants. *Adv Genet* **28**: 99–131
- Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**: 4350–4354
- Volger HG, Heber U (1975) Cryoprotective leaf proteins. *Biochim Biophys Acta* **412**: 335–349
- Volokita M, Somerville CR (1987) The primary structure of spinach glycolate oxidase deduced from the DNA sequence of a cDNA clone. *J Biol Chem* **262**: 15825–15829
- Weiser CJ (1970) Cold resistance and injury in woody plants. *Science* **169**: 1269–1278