Plant Physiol. (1990) 94, 1078–1083
0032-0889/90/94/1078/06/$01.00/0

Received for publication April 20, 1990
Accepted July 2, 1990

Cold Acclimation in *Arabidopsis* and Wheat[1]

A Response Associated with Expression of Related Genes Encoding 'Boiling-Stable' Polypeptides

Chentao Lin, Wei Wen Guo, Everett Everson, and Michael F. Thomashow*

Department of Crop and Soil Sciences (W.W.G., E.E., M.F.T.), Department of Microbiology and Public Health (M.F.T.), Program in Genetics (C.L., M.F.T.), Michigan State University, East Lansing, Michigan 48824-1325

ABSTRACT

Changes in gene expression occur during cold acclimation in a wide variety of plant species. Here we show that a number of the polypeptides encoded by cold-regulated (*cor*) genes of *Arabidopsis thaliana* L. (Heyn) and wheat share the unusual biochemical property that they remain soluble upon boiling in aqueous solution. Further, cDNA cloning in conjunction with Southern and Northern analyses indicate that wheat has a *cor* gene that is related to *Arabidopsis cor47*, a gene encoding a 47 kilodalton 'boiling-stable' COR polypeptide. We suggest it is likely that the boiling-stable COR polypeptides have a fundamental role in plants acclimating to cold temperatures and discuss the possibility that they may act as cryoprotectants.

Cold acclimation is a complex response involving a variety of physical and biochemical changes (18, 20). One of the hallmarks of cold acclimation is increased freezing tolerance; in many species of plants, a period of exposure to low, nonfreezing temperature results in an increased tolerance to freezing temperatures (18, 20). Biochemical changes that have been associated with cold acclimation include alterations in lipid composition, increased sugar and soluble protein content, and the appearance of new isozymes (18, 20, 24). In most cases, the precise role that a given biochemical change has in the cold acclimation process is uncertain. Presumably, some contribute to the overall fitness of the plant for low temperature survival while others have specific roles in bringing about increased freezing tolerance. Indeed, it has been demonstrated that changes in membrane lipid composition can contribute directly to the freezing tolerance of plant cells (24, 25). In addition, there is evidence that proline and many simple sugars (3, 22, 26), as well as certain soluble polypeptides from spinach (30), have cryoprotective effects *in vitro*. Whether these molecules contribute significantly to freezing tolerance *in vivo*, however, remains to be determined.

[1] This work was supported by grants to M. F. T. from the U.S. Department of Agriculture Competitive Research Grants Office (88-37264-3880) and the Michigan Agricultural Experiment Station. W. W. G. was supported in part by a grant to E. E. from the Michigan State Millers Association.

The physical and biochemical changes that occur in plants during cold acclimation could be brought about by preexisting structures and enzymes that undergo alterations in their properties at low temperature. It is also possible, as first proposed by Weiser (31), that cold acclimation involves changes in gene expression. Indeed, recent studies with a variety of plant species have demonstrated that changes in gene expression occur during cold acclimation (11, 27). Little is known, however, about the nature of *co*ld-*r*egulated (*cor*) genes. Are any of the polypeptides encoded by *cor* genes related at the structural or functional level? Do plants have related *cor* genes that are activated during the cold acclimation process? Do *cor* genes have critical roles in freezing tolerance or some other aspect of low temperature survival? Here we begin to address these basic questions for *cor* genes from *Arabidopsis* and wheat.

MATERIALS AND METHODS

Plant Material

Arabidopsis thaliana L. (Heyn) cv Landsberg *erecta* and the winter wheat *Triticum aestivum* L. cv Winoka were grown in controlled environment growth chambers at 21°C either under constant fluorescent light (*Arabidopsis*) or a 14/10 h day/night cycle (wheat); light intensity was approximately 120 μE $m^{-2}s^{-1}$. *Arabidopsis* plants (approximately 2 weeks old) were cold acclimated by transferring them to chambers set at 5°C for 3 d with continuous light. Under these conditions, the LT_{50} of *Arabidopsis* decreases from about −3°C (nonacclimated plants) to about −8°C (cold acclimated plants) (9; S. J. Gilmour, unpublished results). Wheat seedlings (approximately 2 weeks old) were cold acclimated by transferring plants to growth chambers set at either 2 or 7°C for 3 weeks with a 14/10 h day/night cycle. Under these conditions, the LT_{50} of of plants decreased from about −5°C (nonacclimated plants) to either −12°C (when acclimated at 7°C) or −15°C (when acclimated at 2°C) (W. W. Guo, unpublished data).

RNA and DNA Extraction

Poly(A^+) RNA and total DNA were prepared from *Arabidopsis* and wheat as previously described (9, 13).

Preparation and Screening of Wheat cDNA Library

Poly(A$^+$) RNA was isolated from leaves of cold acclimated wheat seedlings and double-stranded cDNA was synthesized essentially as described by Gubler and Hoffman (10). *Eco*RI linkers were added to the cDNAs and the fragments inserted into the *Eco*RI site of lambda ZAP (Stratagene). Recombinant phage were packaged *in vitro* using Packagene (Promega) and transduced into *Escherichia coli* BB4 (Stratagene). Approximately 10^5 primary recombinants were obtained. The library was amplified once and frozen in SM buffer (SM buffer is 100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl [pH 7.5], 0.01% [w/v] gelatin) containing 7% (v/v) DMSO (21).

Plaque lifts were prepared on Nytran membranes (Schleicher and Schuell) using standard methodologies (1, 21). The cDNA insert from pHH7.2 (13; see text) was ^{32}P-labeled by random priming (8). Hybridization was at 60°C in buffer containing 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.5% (w/v) SDS, and 0.25% (w/v) nonfat dry milk. Washes were done at moderate stringency: 2 × SSC, 0.5% (w/v) SDS at 60°C. Phage displaying homology to the pHH7.2 probe were plaque purified and the cDNA inserts 'subcloned' in pBluescript SK$^-$ as described by the supplier (Stratagene).

Southern and Northern Analysis

Southern and Northern blots were prepared on Nytran membranes using standard methods (1, 21) as previously described (13). ^{32}P-Labeling of probes and Southern hybridization and wash conditions were the same as those used in screening the cDNA library. Northern hybridization and wash conditions were as described (13).

Detection of Boiling-Stable COR Polypeptides

Poly(A$^+$) RNA was translated *in vitro* using a rabbit reticulocyte lysate system (Promega) with [^{35}S]methionine as radiolabel. Translation products were diluted with 5 volumes of 50 mM Tris-HCl (pH 7.0), the samples placed in a boiling water bath for 10 min, and the insoluble material removed by centrifugation in an Eppendorf microfuge (15 min). Polypeptides that remained soluble were precipitated with 7 volumes of acetone and collected by centrifugation in a microfuge (15 min). The pelleted material was suspended in loading buffer (10% [v/v] glycerol, 0.01% [w/v] bromphenol blue, 2% [w/v] SDS, 60 mM Tris-HCl [pH 6.9], 100 mM dithiothreitol) and fractionated by SDS-PAGE (17).

Hybrid-Arrest Translation Experiments

Hybrid-arrest translations were done as described (15) using 1 μg poly(A$^+$) RNA and 0.5 μg single-stranded DNA. Single-stranded DNA was prepared from pHH7.2 (the antisense strand for the *cor47* gene is produced; see text) and the cloning vector pBluescript SK$^-$ by phage rescue as described by Vieira and Messing (29).

RESULTS

Cold Acclimation in *Arabidopsis* and Wheat is Associated with Accumulation of Transcripts Encoding Boiling-Stable Polypeptides

We (9, 13, 28) and others (16) have established that *Arabidopsis thaliana* becomes more frost tolerant when exposed to low, nonfreezing temperatures and that changes in gene expression occur with cold acclimation. In particular, we have observed that mRNAs encoding polypeptides of 160, 47, 24, and 15 kD consistently accumulate in cold acclimated plants (9, 28); accumulation of the COR160 and COR47 polypeptides were also detected in such plants (9, 28) (the experiments were not designed to detect the COR24 and COR15 polypeptides). As a step toward determining the functions of these COR polypeptides, we have begun to characterize their physical properties.

We have found that all four of the major COR polypeptides share an unusual biochemical property: they remain soluble upon boiling in aqueous solution. This was shown as follows. Poly(A$^+$) RNA isolated from both cold acclimated and nonacclimated plants was translated *in vitro* and the polypeptide products were fractionated either directly by SDS-PAGE or

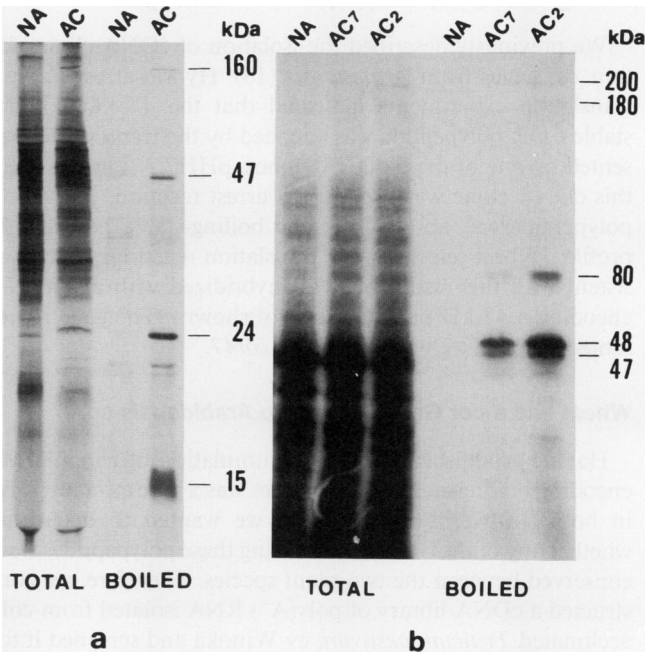

Figure 1. Accumulation of transcripts encoding boiling-stable polypeptides in cold acclimated *Arabidopsis* and wheat. Poly(A$^+$) RNA isolated from cold acclimated and nonacclimated *Arabidopsis* (a) and wheat (b) was translated *in vitro* and the polypeptide products were either fractionated directly by SDS-PAGE (TOTAL) or were first boiled and treated as described in "Materials and Methods" and then fractionated (BOILED). The TOTAL and BOILED samples represent approximately 5 and 25 μL of the *in vitro* translation products, respectively. Film exposures were for approximately 3 d. The gels in (a) and (b) were 15 and 10% (w/v) polyacrylamide, respectively. Lanes in (a) are: NA, nonacclimated; AC acclimated. Lanes in (b) are: NA, nonacclimated; AC7, acclimated at 7°C; AC2, acclimated at 2°C.

were first boiled, centrifuged to remove insoluble material, and then fractionated by SDS-PAGE (Fig. 1a). As anticipated, the boiling treatment resulted in precipitation of the majority of the polypeptides translated from the RNA isolated from either the acclimated or nonacclimated plants. However, the 160, 47, 24, and 15 kD COR polypeptides remained soluble. In addition, transcripts encoding boiling-stable polypeptides of about 18, 20, and 21 kD appeared to increase in the acclimated plants.

The accumulation of *cor* transcripts encoding boiling-stable polypeptides is not unique to *Arabidopsis*; it also occurs in cold acclimated *Triticum aestivum* cv Winoka, a cultivar of winter wheat. Poly(A^+) RNA was isolated from cold acclimated and nonacclimated plants, the samples were translated *in vitro,* and the polypeptides were assayed for boiling-stability as described above. Again, boiling the *in vitro* translation products resulted in precipitation of nearly all of the polypeptides in both the acclimated and nonacclimated samples (Fig. 1b). However, as with *Arabidopsis, cor* mRNAs encoding boiling-stable polypeptides were found to accumulate in the cold acclimated plants. In this case, boiling-stable COR polypeptides of about 200, 180, 80, 48, 47 (Fig. 1b), and 17 kD (not shown) were observed.

Identification of a cDNA Clone Encoding the 47 kD Boiling-Stable COR Polypeptide of *Arabidopsis*

We previously described the isolation of cDNA clones for four *cor* genes from *Arabidopsis* (13). Hybrid-arrest *in vitro* translation experiments indicated that the 47 kD boiling-stable COR polypeptide was encoded by the transcript represented by one of these cDNA clones, pHH7.2 (Fig. 2); when this cDNA clone was used in the arrest reaction, the 47 kD polypeptide was absent from the boiling-stable polypeptide profile. Hybrid-select *in vitro* translation reactions were consistent with this result; pHH7.2 hybridized with an mRNA encoding a 47 kD polypeptide (not shown). The gene represented by pHH7.2 was designated *cor47*.

Wheat has a *cor* Gene Related to *Arabidopsis cor47*

Having established that the accumulation of *cor* mRNAs encoding boiling-stable polypeptides was a common response in both *Arabidopsis* and wheat, we wanted to determine whether any of the *cor* genes encoding these polypeptides were conserved between the two plant species. Therefore, we constructed a cDNA library of poly(A^+) RNA isolated from cold acclimated *Triticum aestivum* cv Winoka and screened it for clones that would hybridize with *Arabidopsis cor47* (the insert from pHH7.2 was used as the probe). Such clones were detected, and one, pWG1, was examined further.

Southern analysis indicated that the DNA sequence homology between pWG1 and pHH7.2 mapped to the middle region of the pHH7.2 cDNA insert, primarily to the section between the *Xba*I and *Kpn*I restriction sites (Fig. 3). DNA sequence analysis of pHH7.2 indicates that this region is within the coding sequence of the COR47 polypeptide (S. J. Gilmour, M. F. Thomashow, unpublished data). Northern analysis was then conducted to determine whether the wheat

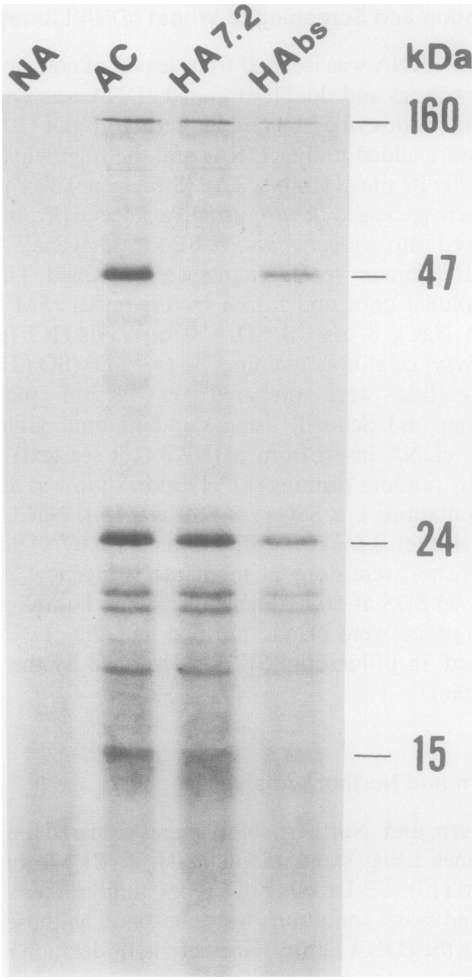

Figure 2. Hybrid-arrest translation assays indicating *Arabidopsis* cDNA clone pHH7.2 represents the *cor* transcript encoding the 47 kD boiling-stable COR polypeptide. Poly(A^+) RNA was isolated from cold acclimated *Arabidopsis* and either translated *in vitro* without hybrid-arrest (AC) or after hybrid-arrest with single-stranded probes prepared from either pHH7.2 (HA7.2) or the cloning vector pBluescript SK^- (HAbs) (Stratagene). Poly(A^+) RNA isolated from nonacclimated *Arabidopsis* was also translated *in vitro* without hybrid arrest (NA). Translation products from each reaction were assayed for boiling-stable polypeptides as described in "Materials and Methods." The amount of radioactivity loaded in lanes AC and HA7.2 was approximately three times greater than that loaded in lane HAbs. Film exposure was for approximately 3 d.

gene represented by pWG1 was indeed a *cor* gene (*i.e.* was cold-regulated). The results indicated that it was; transcripts homologous to pWG1 were only present in cold acclimated plants (Fig. 4). Interestingly, transcripts of four different lengths were observed: 3.3, 1.5, 1.4, and 0.8 kb. Whether the different transcripts originate from the same gene or different genes remains to be determined. It is also noteworthy that the lengths of the major transcripts detected with pWG1 in wheat (1.5 and 1.4 kb) were about the same as that detected with pHH7.2 in *Arabidopsis* (1.4 kb) (13). Thus, the wheat gene represented by pWG1 may encode a polypeptide(s) similar in mass to that encoded by *Arabidopsis cor47*. The observation

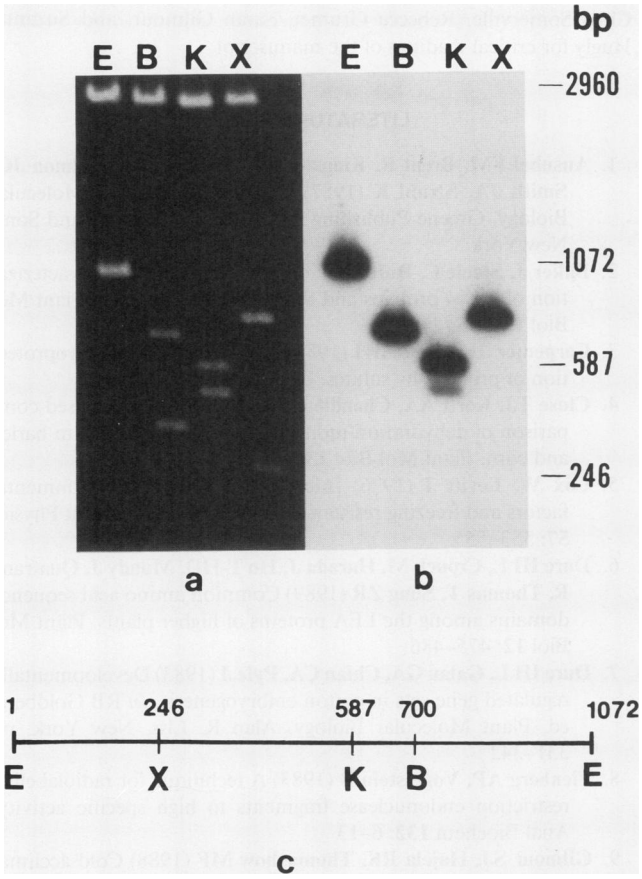

Figure 3. DNA homology between *Arabidopsis cor47* and a wheat gene represented by pWG1. (a) Agarose gel electrophoresis of restriction fragments generated by digesting pHH7.2 with: E, *Eco*RI; B, *Eco*RI plus *Bam*HI; K, *Eco*RI plus *Kpn*I; and X, *Eco*RI plus *Xba*I. (b) Southern blot of the gel depicted in (a) hybridized at moderate stringency (see "Materials and Methods") with the cDNA insert contained in pWG1. (c) Restriction map of the cDNA insert in pHH7.2. The indicated restriction sites are: E, *Eco*RI; X, *Xba*I; K, *Kpn*I; and B, *Bam*HI. Distances of restriction sites from the left *Eco*RI site, given in basepairs (bp), was determined by DNA sequencing (S. J. Gilmour, M. F. Thomashow, unpublished results).

of boiling-stable polypeptides of about 47 and 48 kD in the *in vitro* translations of RNA isolated from cold acclimated wheat (Fig. 1b) is consistent with this notion.

DISCUSSION

The data presented indicate that several of the *cor* transcripts from *Arabidopsis* and wheat encode polypeptides that share the unusual biochemical property of remaining soluble upon boiling in aqueous solution. In addition, the data indicate that at least one wheat *cor* gene is related to an *Arabidopsis cor* gene, specifically *cor47*. Given the evolutionary distance between *Arabidopsis* and wheat, it is probable that the accumulation of *cor* mRNAs encoding boiling-stable polypeptides will prove to be a common low temperature response in plants. Indeed, initial studies indicate that the response also occurs in both spinach and *Solanum acaule* (C Lin, unpublished results). It would also seem likely that genes related to *Arabidopsis cor47* will be found in additional plant species. Future studies will determine whether this is true and whether any of the other *Arabidopsis* or wheat *cor* genes have counterparts in other plants.

The finding that certain *cor* genes encode boiling-stable polypeptides is significant in terms of their potential function. First, it strongly suggests that the expression of these genes in response to low temperature is not fortuitous. If it were, it would be improbable that so many would encode polypeptides sharing the same unusual property, boiling-stability, and that such a large proportion of the boiling-stable polypeptides would be cold-regulated. Further, it would seem unlikely that wheat would have a gene related to one of the *Arabidopsis cor* genes and that it too would be cold-regulated. In our view, the more likely situation is that the *cor* genes encoding boiling-

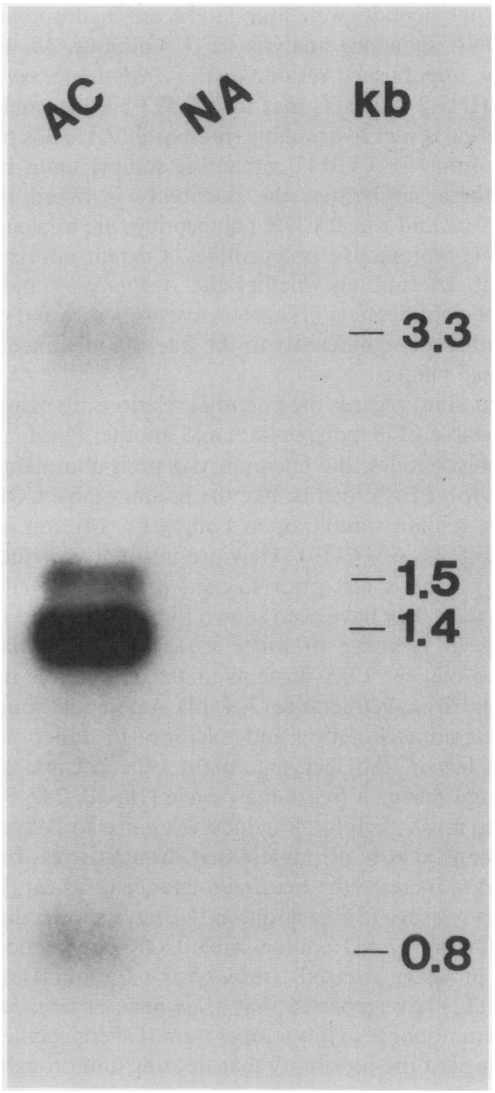

Figure 4. Northern analysis indicating that pWG1 represents a wheat *cor* gene. Poly(A$^+$) RNA was isolated from nonacclimated (NA) and cold acclimated (AC) wheat. Samples were fractionated on denaturing formaldehyde-agarose gels and Northern blots prepared and hybridized with ^{32}P-labeled insert from pWG1. The approximate lengths of the transcripts are indicated in kilobases (kb).

stable polypeptides have a fundamental role in plants acclimating to cold temperatures and that the boiling-stable nature of the polypeptides is reflective of their function.

What function(s) might the boiling-stable COR polypeptides have? One possibility is suggested by the work of Volger and Heber (30). These investigators have reported that cold acclimated spinach synthesizes polypeptides that, on a molar basis, are greater than 1000 times more effective in protecting thylakoid membranes against freezing damage (*in vitro*) than are known low mol wt cryoprotectants such as sucrose and glycerol. Intriguingly, these polypeptides appear to have distinctive features in common with the *Arabidopsis* and wheat COR polypeptides described here: notably cold-regulation (the polypeptides were detected in cold acclimated plants but not in nonacclimated plants) and heat-stability (they were not irreversibly denatured by high temperatures). In addition, the spinach polypeptides were found to be very hydrophilic. Likewise, DNA sequence analysis (S. J. Gilmour, M. F. Thomashow, unpublished results) of the *Arabidopsis cor* cDNA clone pHH7.2 indicates that the 47 kD boiling-stable COR polypeptide is very hydrophilic (presumably it is this property that accounts for COR47 remaining soluble upon boiling). Given these similarities, the possibility is raised that the *Arabidopsis* and wheat COR polypeptides are analogs of the spinach cryoprotective polypeptides. Current efforts are directed at determining whether the *Arabidopsis* and wheat COR polypeptides have cryoprotective properties and whether they contribute significantly to the freezing tolerance of cold acclimated plants.

A final point regards the potential relationship between the boiling-stable COR polypeptides and another family of heat-stable polypeptides, the late-embryogenesis-abundant (LEA) proteins (6). LEA proteins, like the boiling-stable COR polypeptides, remain soluble upon boiling (4, 14) and are very hydrophilic (4, 6, 14, 19). They are normally produced late in embryogenesis just prior to seed desiccation (7) and in certain cases, they have been shown to be synthesized in plant seedlings in response to water stress (4, 19). It has been suggested that the LEA proteins might have a role in desiccation and drought tolerance (2, 4, 14). A potential connection between cold acclimation and tolerance to desiccation and drought lies in the fact that plant cells become severely dehydrated during a freeze-thaw cycle (18, 20, 24). Freezing tolerance must, therefore, include tolerance to dehydration. In this context it is of interest that drought stress has been observed to increase the freezing tolerance of wheat (23), rye (23), and cabbage (5). In addition, we have shown that transcripts for the 47 kD boiling-stable COR polypeptide accumulate in water stressed *Arabidopsis* (13) and Hahn and Walbot (12) have reported that a *lea* gene of rice, *rab21,* is induced in response to low temperature. Taken together, these results suggest the possibility that freezing and drought tolerance involve related genetic mechanisms that include the action of *cor* and *lea* genes. Future studies will be directed at testing the validity of this notion.

ACKNOWLEDGMENTS

We thank Peter Chandler for discussions regarding the boiling-stable nature of the barley dehydrins prior to publication and thank Chris Somerville, Rebecca Grumet, Sarah Gilmour, and Suzanne Hugly for critical readings of the manuscript.

LITERATURE CITED

1. **Ausubel FM, Brent R, Kingston RE, Moore DD, Seidmon JG, Smith JA, Struhl K** (1987) Current Protocols in Molecular Biology. Greene Publishing Associates, John Wiley and Sons, New York
2. **Baker J, Steele C, Dure III L** (1988) Sequence and characterization of 6 *Lea* proteins and their genes from cotton. Plant Mol Biol **11:** 277–291
3. **Carpenter JF, Crowe JH** (1988) The mechanism of cryoprotection of proteins by solutes. Cryobiology **25:** 244–255
4. **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
5. **Cox W, Levitt J** (1976) Interrelation between environmental factors and freezing resistance of cabbage leaves. Plant Physiol **57:** 553–555
6. **Dure III L, Crouch M, Harada J, Ho T-HD, Mundy J, Quatrano R, Thomas T, Sung ZR** (1989) Common amino acid sequence domains among the LEA proteins of higher plants. Plant Mol Biol **12:** 475–486
7. **Dure III L, Galau GA, Chlan CA, Pyle J** (1983) Developmentally regulated gene sets in cotton embryogenesis. *In* RB Goldberg, ed, Plant Molecular Biology. Alan R. Liss, New York, pp 331–342
8. **Fienberg AP, Vogelstein B** (1983) A technique for radiolabeling restriction endonuclease fragments to high specific activity. Anal Biochem **132:** 6–13
9. **Gilmour SJ, Hajela RK, Thomashow MF** (1988) Cold acclimation in *Arabidopsis thaliana.* Plant Physiol **87:** 745–750
10. **Gubler U, Hoffman BJ** (1983) A simple and very efficient method for generating cDNA libraries. Gene **25:** 263–269
11. **Guy CL** (1990) Cold acclimation and freezing stress tolerance: Role of protein metabolism. Annu Rev Plant Physiol Plant Mol Biol **41:** 187–223
12. **Hahn M, Walbot V** (1989) Effects of cold-treatment on protein synthesis and mRNA levels in rice leaves. Plant Physiol **91:** 930–938
13. **Hajela RK, Horvath DP, Gilmour SJ, Thomashow MF** (1990) Molecular cloning and expression of *cor* (*co*ld-*r*egulated) genes in *Arabidopsis thaliana.* Plant Physiol **93:** 1246–1252
14. **Jacobsen JV, Shaw DC** (1989) Heat-stable proteins and abscisic acid action in barley aleurone cells. Plant Physiol **91:** 1520–1526
15. **Jagus R** (1987) Hybrid selection of mRNA and hybrid arrest of translation. Methods Enzymol **152:** 567–572
16. **Kurkela S, Franck M, Heino P, Lång V, Palva ET** (1988) Cold induced gene expression in *Arabidopsis thaliana* L. Plant Cell Rep **7:** 495–498
17. **Laemmli UK** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685
18. **Levitt J** (1980) Responses of Plants to Environmental Stress. Chilling, Freezing, and High Temperature Stresses, Ed 2. Academic Press, New York
19. **Mundy J, Chua N-H** (1988) Abscisic acid and water stress induce the expression of a novel rice gene. EMBO J **7:** 2279–2286
20. **Sakai A, Larcher W** (1987) Frost Survival of Plants. Responses and Adaptations to Freezing Stress. Springer-Verlag, New York
21. **Sambrook J, Fritsch EF, Maniatis T** (1989) Molecular Cloning. A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
22. **Santarius KA** (1973) The protective effect of sugars on chloro-

plast membranes during temperature and water stress and its relationship to frost, desiccation and heat resistance. Planta **113:** 105–114

23. **Siminovitch D, Cloutier Y** (1983) Drought and freezing tolerance and adaptation in plants: Some evidence of near equivalences. Cryobiology **20:** 487–503
24. **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
25. **Steponkus PL, Uemura M, Balsamo RA, Arvinte T, Lynch DV** (1988) Transformation of the cryobehavior of rye protoplasts by modification of the plasma membrane lipid composition. Proc Natl Acad Sci USA **85:** 9026–9030
26. **Strauss G, Hauser H** (1986) Stabilization of lipid bilayer vesicles by sucrose during freezing. Proc Natl Acad Sci USA **83:** 2422–2426
27. **Thomashow MF** (1990) Molecular genetics of cold acclimation in higher plants. Adv Genet "in press"
28. **Thomashow MF, Gilmour SJ, Hajela R, Horvath D, Lin C, Guo W** (1990) Studies on cold acclimation in *Arabidopsis thaliana*. *In* AB Bennett, SD O'Neill, eds, Horticultural Biotechnology. Wiley-Liss, New York, pp 305–314
29. **Vieira J, Messing J** (1987) Production of single-stranded plasmid DNA. Methods Enzymol **153:** 3–11
30. **Volger HG, Heber U** (1975) Cryoprotective leaf proteins. Biochim Biophys Acta **412:** 335–349
31. **Weiser CJ** (1970) Cold resistance and injury in woody plants. Science **169:** 1269–1278