A MOLECULAR GENETIC STUDY OF COLD ACCLIMATION IN *ARABIDOPSIS THALIANA*

By

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ABSTRACT

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Changes in gene expression have been suggested to have roles in plant cold acclimation. The functions of *cor* (cold-regulated) genes, however, remains to be determined. Plant *cor* genes and their products were studied using *Arabidopsis* as a model system. Cold acclimation of *Arabidopsis* was found to be associated with the accumulation of *cor* gene transcripts encoding polypeptides that share the unusual property of remaining soluble upon boiling in aqueous solution. Genes encoding three "boiling-stable" polypeptides, COR160, COR47, and COR15 were shown to be represented by three *cor* cDNA clones pHH28, pHH7.2, and pLCT10, respectively. The polypeptide COR15 and cDNA clones encoding it were further characterized. It was found that COR15 has potent cryoprotective activity *in vitro*: it was 10^6 fold more effective than sucrose, and 10^2-10^3 fold more effective than other proteins tested in protecting lactate dehydrogenase against freeze inactivation. DNA sequence analysis of the cDNA clone pLCT10 indicated that the N-terminal amino acid sequence of COR15 resembles chloroplast transit peptides. Immunological studies further demonstrate that COR15 is indeed targeted to the chloroplast and processed to a mature form of approximately 9 kDa. Attempts to create and analyze transgenic *Arabidopsis* plants that overexpress or underexpress *cor15* are discussed.

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LITERATURE REVIEW

I. INTRODUCTION

Freezing is a stress of widespread occurrence to plants; for about 50% of the earth's land, the average minimum temperature is below -10°C (see Sakai and Larcher 1987). Freezing stress represents the most important environmental constraint limiting the distribution of plants (Parker, 1963). Together with drought and salt stress, freezing also represents one of the major limiting factors in productivity of agriculture crops. For example, if winter wheat and rye could be made 2°C more cold hardy, these new cultivars could replace much of the spring wheat and rye grown in North America and territories of the former Sovite Union to increase the crop yields by 25 to 40 percent (see Salisbury and Ross 1985).

Through evolution, plants have developed mechanisms to allow them to survive different environmental stresses occuring in the habitat in wild they originated. Most plant species from temperate and alpine regions can increase their resistance to freezing stress via a process triggered by exposure to low but non-freezing temperature (see Levitt, 1980; Sakai and Larcher, 1987; Thomashow, 1990). This process is termed cold acclimation or cold hardening. Plant cold acclimation can be of such manifestation that some acclimated woody plants like dogwood can readily survive

liquid nitrogen temperature of -196^{o}C. These same plants, when actively growing and nonacclimated, are severely injured or killed by only -3^{o}C (Weiser, 1970). Herbaceous plants can also attain a certain level of cold hardiness. Non-acclimated wheats are killed upon freezing to about -5^{o}C while acclimated ones can survive the temperature down to -20^{o}C. Non-acclimated *Arabidopsis thaliana* are killed at about -3^{o}C but they can survive a freezing temperature of -10^{o}C after cold acclimation (Gilmour *et al* 1988).

For decades, the study of plant cold acclimation has been focused on two primary questions: the mechanism(s) of freezing injury, and the biochemical and physiological changes that occur during cold acclimation. Enormous amounts of information have been generated which have improved our understanding of plant cold acclimation. However, our knowledge about the mechanism(s) of cold acclimation and freezing resistance is still far from satisfactory either for our scientific curiosity to understand its nature or for the practicality of achieving crop improvment. More recently, the development of molecular biology has brought a new approach to the study of cold acclimation (see Thomashow, 1990). This approach is based on an early idea that variation in hardiness among plant species and seasonal variation within the tissues of a single species have a genetic basis (Weiser 1970). By determination and characterization of the genes whose expression are altered by low temperatures, this new approach may provide insight into which biochemical and physiological changes in cold acclimation are directly responsible for altered freezing resistance in plants.

II. FREEZING INJURY

Freezing injury may occur in all plants, in contrast to chilling injury that only occurs in tropical and subtropical plants. Among the more commonly recognized types of freezing injury are spring and autumn frost damage to tender annuals, flowers and fruits, crown kill of winter cereals and herbaceous perennials, die-back in citrus, sun scald of tree trunks, and frost cankers and burls in conifers and hardwoods. Upon freezing-injury, the plant tissues appear decolorized or bleached, and the tissues shrink or dieback. When injuries occur to meristematic and incompletely differentiated tissue, the organs that subsequently develop would exhibit distortions, stunting or fragmentation. Freezing damage can also delay the onset of various stages of development.

By definition, freezing is a process in which the crystallization of ice occurs. Depending on the rate of cooling, freezing can happen in two markedly distinct locations within the plant tissues: extracellular or intracellular. If the cooling rate is slow, as most frequently occurs in nature ($< 1\text{-}2^{o}C$/hr), freezing is most likely to occur extracellularly (Mazur, 1969; Steffen *et al* 1989). It has been suggested that ice normally crystallizes first in the large vessels, probably because of their large diameter which does not favor supercooling, and their dilute sap which also contributes to the higher freezing point (Olien, 1967). In almost every case, ice crystals will spread from the initial site throughout the extracellular region of the tissue and continue to grow if the duration of subzero temperature is extended. At a slow cooling rate, ice crystals are barred from entering the cell by the plasma membrane surrounding each cell, where intracellular ice nucleation is usually not favored. Consequently, ice from vessels only spread through the intercellular spaces. On the other hand, if the cooling rate is moderately rapid ($5\text{-}20^{o}C$/hr), freezing may occur intracellularly. At temperatures that

occur in nature, intracellular freezing is considered to be universally and instantaneously lethal to plant cells (Burke *et al.*, 1976; Sakai and Larcher, 1987). It should be noted that the rapid cooling rate causing intracellular freezing is seldom found in nature (Levitt, 1980).

Since it is hard to know whether a cell surving a freeze-thaw cycle has been "injured", research into the processes involved in freezing injury in plants has always been directed primarily towards the elucidation of causes of cell death. Of the many hypotheses and theories that have been proposed over the years, none have proved entirely satisfactory (see Sakai and Larcher, 1987). The main reason for this is probably lies in the diversity of aspects depending upon species, state of acclimation, and the condition of freezing, so that one general mechanism can not be singled out to be responsible for cell death or survival. It is, however, generally accepted that intracellular ice formation always causes direct injury leading to the death of the frozen cells, and extracellular freezing results in indirect physical injury to the protoplasm. Indirect injury may have different manifestations such as mechanical damage, air expulsion, freeze smothering, and freeze dehydration (Levitt, 1980). Among these, freeze-induced dehydration may be the most well documented. Freeze dehydration may also be the most important mechanism of freezing injury to plant cells because of the fact that intracellular freezing is rarely observed in nature (Levitt, 1980).

As mentioned before, the plasma membrane is an effective barrier for ice crystals to invade the protoplast. When water in the intercellular spaces freezes gradually, the extracellular water potential declines rapidly compared to the intracellular water potential. First, the absolute value for osmotic potential of the extracellular spaces decreases (i.e. it becomes more negative) as a result of the freeze

-induced concentration of salt. Secondly,as the temperature drops, pressure potential (or vapor pressure), another component of water potential, of the ice outside of the cell decreases faster than the vapor pressure of liquid water inside the cell. A water potential gradient is thus established, with liquid water moving down the gradient to the extracellular spaces. It has been calculated that the decline in the water potential of ice with decreasing temperature can be as large as -1.16 MPa/^{o}C (see Guy, 1990). This decline in water potential outside of the cell can create a very steep gradient across the plasma membrane, considering the osmotic potential for most plant saps lie between -0.4 and -2.0 MPa (Salisbury and Ross, 1985). Consequently, protoplasts at equilibrium with extracellular ice will dehydrate in a strict temperature-dependent relationship.

One of the extreme consequences resulting from extracellular freezing is "frost plasmolysis." Cells killed by extracellular freeze characteristically show frost plasmolysis, a contraction of the dead protoplast leaving a large space between it and its cell wall (see Levitt 1980). Frost plasmolysis is due the osmotic contraction of the cell during extracellular ice formation and the inability of the dead protoplast to reabsorb the water formed in the intercellular spaces on thawing of extracellular ice. As a result, the cell wall expands back to nearly its original shape, while the dead protoplast remains contracted. This would seem to indicate that the injury had occurred during the freezing and the cells were nonfunctional by the time the ice began to thaw. Although it is not clear exactly how freeze dehydration can cause cell death, various kinds of deteriorating effects of freeze-dehydration have been proposed (see Levitt, 1980, Steponkus, 1990).

As mentioned above, the plasma membrane plays a central role in cellular behavior during freezing. As early as 1912 Maximov suggested that disruption of cellular membrane, particularly the plasma membrane, was the primary form of

freezing injury in plants, and the subsequent studies have supported this view (see Levitt, 1980; Steponkus, 1990). It has been proposed that freeze-induced dehydration results in two different forms of injury depending on the temperature and the extent of cell dehydration (Steponkus *et al* 1982; Steponkus and Lynch 1989; Steponkus 1990). The first type of freezing injury in the plasma membrane is "expansion-induced lysis", which occurs at a relatively high (-3 to -7^{o}C) temperature. In contrast, when cells are cooled to a lower temperature (e.g. -10^{o}C) and subjected to a greater extent of cell dehydration (e.g. about 90% of the osmotically active water is removed), the protoplasts are osmotically unresponsive during thawing. This second form of injury is referred to as "loss of osmotic responsiveness." Although both forms of injury occur as a result of cell dehydration, the mechanisms of injury are vastly different.

It was suggested that the elastic expansion/contraction of the plasma membrane is limited to changes in area of about 2-3% (Klosson and Krause, 1981). For larger changes in the surface area of the plasma membrane, as usually occurs during cell dehydration, membrane material is either deleted from the membrane during osmotic contraction or incorporated into the membrane during osmotic expansion. The deletion has been observed as endocytotic vesicles in nonacclimated rye protoplast by computer -enhanced microscopy (Dowgert and Steponkus, 1984) and electron microscopy (Gordon-Kamm and Steponkus, 1984a; Jonhson-Flanagan and Singh, 1986). The deleted membrane materials formed during osmotic contraction do not re-enter the membrane during subsequent osmotic expansion. As a result, during cell rehydration, the irreversible deletion of the membrane leads to an intolerable osmotic pressure and the protoplast bursts. In contrast, when protoplast isolated from cold acclimated plants were subjected to similar osmotic contraction, the plasma membrane formed exocytotic extrusions instead of endocytotic vesicules. The important difference between exocytotic extrusion and endocytotic vesiculation is that the membrane material of

exocytotic extrusions remains associated with the plasma membrane and is reincorporated during rehydration. As a result, the protoplasts can swell back to their original size rather than lyse as is the case with endocytotic vesiculation.

The second type of plasma membrane injury has been shown to occur at -10°C and below. At these lower temperatures, the dehydration experienced by the cell is more severe than freezing at -5°C, and results in the "loss of osmotic responsiveness" because the plasma membranes lose their semipermeability during osmotic contraction. A conceivable consequence of this type of injury is the frost plasmolysis described before. The loss of osmotic responsiveness is associated with several changes in ultrastructure of the plasma membrane, including the appearance of lateral phase separations and lamellar-to-hexagonal II phase transitions (Gordon-Kamm and Steponkus, 1984a,b; Pearce and Willison, 1985; Johnson-Flanagan and Singh, 1986; Singh *et al.*, 1987). Again, the second type of injury and the ultrastructural changes associated with it are greatly reduced in cold acclimated cells. The membrane ultrastructural changes and the loss of osmotic responsiveness are suggested to be the consequences of freeze-induced dehydration rather than the exposure to subzero temperature *per se*. This is indicated by the fact that the same injury can be induced by subjecting protoplasts to equivalent osmotic-dehydration (5.4 osm sorbitol) in the absence of ice formation. In addition, protoplast suspensions can be supercooled to temperatures of -5°C to -15°C without deliterious effects (Gordon-Kamm and Steponkus, 1984b; Dowgert *et al.*, 1987).

It has been suggested that dehydration increases the membrane liquid-crystalline to solid-gel phase transition temperature (see Steponkus and Lynch, 1989). One consequence of such crystalline-to-gel phase transition is the occurrence of laterally separated regions with clusters of intramembrane particles enriched in some areas and smooth, particle-depleted surfaces in other areas of the membrane, a characteristic

feature of the lateral phase transition observed in freeze-injured membranes. A dehydration-induced crystalline-to-gel phase transition also results in demixing of the lipid mixture and localized enrichment of nonbilayer-forming lipids such as phosphatidylethanolamine which, upon further dehydration, would undergo lamellar-to-hexaganol II phase transition. The hexaganol II phase is not semipemeable and not responsive to osmotic changes.

Besides the direct injury to plasma membranes, both freeze-induced dehydration and freezing temperature also impose deleterious effects to other organelles and macromolecules of the cell. For example, the chloroplast has been regarded as another major site of injury by freezing (Levitt, 1980; Hincha and Schmitt, 1991). When leaves are frozen to lethal temperatures, photosynthesis is irreversibly damaged (Krause *et al.*, 1988; Steflen *et al.*, 1989). It has been shown that both freezing and severe dehydration can cause rupture of thylakoid membranes as indicated by the release of the lumen protein plastocyanin (Hincha *et al.*, 1987; Hincha *et al* 1989). When spinach leaves were frozen at -6^{o}C and thawed slowly, more than 90% of the plastocyanin was released from the thylakoid lumen. Cold acclimation can prevent freeze-induced or dehydration-induced leakage of the plastocyanin. It is not clear what mechanism is responsible for the freeze-induced rupture of chloroplast membranes. Neither is it known how cold acclimation can prevent this type of injury.

Another form of freezing injury is the direct damage to protein molecules as many proteins are freeze labile (Levitt, 1980). Many enzymes lose their activity after freezing and thawing, such as lactate dehydrogenase, catalase, phosphofructokinase, malate dehydrogenase, pyruvate kinase, and ATPase (Carpenter and Crowe, 1988). Protein structures may undergo a number of changes at low temperature or in the dehydrated environment. First, the quaternary structure may be altered leading to dissociation of a large protein complex into monomeric subunits (Markert, 1965). For

example, all of the microtubules in the green alga (*Losterium ehrenbergii*) depolymerize after only 5 min at 0°C (Hogetu, 1986), and the coupling factor CF1 is released from the chloroplast ATPase complex by either freezing or hypertonic conditions (Santarius, 1984, Hincha and Schmitt, 1985). Secondly, the tertiary structure of some proteins may be changed during freezing or dehydration (Brandts, 1967), and the unfolded molecules may aggregate by forming physical bonds (salt bridge, H bond, hydrophobic interactions, S-S bonds, etc) between the newly exposed active chemical groups that were previously buried within the folded molecules. Freeze-induced protein aggregation or precipitation was noticed more than 80 years ago by Gorke (Gorke, 1906), when he found that proteins in the sap expressed from nonacclimated cereal leaves and other plants precipitated upon a freeze-thaw cycle. The more resistant the plant to freezing injury, the lower was the temperature required to precipitate the proteins. Gorke explained freezing injury as a precipitation of the protoplasmic proteins due to the increased concentration of the cell salts that occurs during freezing. Finally, the freezing or dehydration damage to cellular membrane systems has profound effects on the protein molecules previously associated with these membranes or compartmented within organelles. This has been clearly shown by observation on one of the ultrastructural changes in the plasma membrane described previously, lateral phase separation, where some part of the membranes is depleted of membrane protein particles upon freeze-induced dehydration. On the other hand, it has been shown that the leakage of stroma or thylakoid lumen proteins such as coupling factor CF1 and plastocyanin is associated with the inactivation of photophosphorylation and the irreversible damage to photosynthesis during freezing (Hincha *et al* 1987).

It can be concluded that freezing injury to plant cells can be the consequence of either intracellular freezing which is always lethal, or the extracellular freezing that is pontentially tolerable. Extracellular freezing of plant tissues leads to impairment of the

structure and function of plasma membranes, chloroplasts, and other cellular components. Both freezing temperatures and freeze-induced dehydration result in changes in and damage to cellular structures and enzymes. Therefore, it was suggested that when plants become cold acclimated they must have developed both intracellular -freeze avoidance and extracellular-freeze tolerance mechanisms to survive the subsequent freezing environment (Levitt, 1980). Although the above notion is now generally accepted, the detaiils of the mechanisms involved are still controvertial (see Levitt, 1980; Sakai and Larch, 1987; Steponkus, 1990; Guy, 1990).

II. BIOCHEMISTRY OF COLD ACCLIMATION

Research on the biochemistry of cold acclimation has been informative in terms of our understanding about mechanism(s) responsible for the increased freezing tolerance of acclimated plants. However, the majority of these studies have been restricted to either correlative studies of gross changes in certain types of molecules during cold acclimation or comparative studies of these changes in species or varieties of different hardiness (Levitt, 1980). Unfortunately, these studies have not significantly improved our understanding of how cold acclimation leads to increased freezing tolerance in plants. More recently, biochemical studies have been directed more toward direct cause and effect analysis and most progress has been made in membrane lipid studies (see Stenpokus, 1990).

A. Membrane lipid changes during cold acclimation

Early studies on membrane lipid change during cold acclimation were restricted to lipid analysis of whole tissue extracts or crude membrane fractions rather than

plasma membrane *per se*. The improvement of techniques for isolating purified plasma membrane fractions made possible the more direct studies of the alteration in the lipid composition of the plasma membrane during cold acclimation. By comprehensive analysis of more than 50 lipid species of the plasma membrane from cold acclimated and nonacclimated rye leaves, it has been found that cold acclimation alters the proportion of virtually every lipid component; although there are no lipid species that are unique to cold acclimated or nonacclimated plasma membranes (see Lynch and Steponkus, 1987). Following cold acclimation, free sterols increase about 25%, while the steryl glycosides and acylated steryl glycoside decrease about 30% and 25% respectively. The phospholipid content of the plasma membrane also increases for about 25% following cold acclimation. Two major phospholipids, PC (phosphatidylcholine) and PE (phosphatidylethanolamine) increase from 15 to 20 mol % and 11 to 16 mol %, respectively. In addition, the levels of diunsaturated fatty acid species (18:2/18:2, 18:2/18:3, 18:3/18:3) of PC and PE double in cold acclimated cells.

As described in the previous section, the cryobehavior of the plasma membrane from cold acclimated and nonacclimated rye cells is different. Freeze-induced osmotic contraction result in endocytotic vesiculation and exocytotic extrusions in the plasma membranes of nonacclimated and cold acclimated cells, respectively. In addition, the dehydration-induced lamella to hexagonal II phase transition occurs only in plasma membranes of nonacclimated cells but not in plasma membrane of cold acclimated cells (Gordon-Kamm and Steponkus, 1984b). The causal relationship between the changes in membrane lipid druing cold acclimation and the cryobehavior of plasma membranes has been demonstrated by Steponkus and colleagues in an elegant series of experiments (Steponkus *et al.*, 1988; Steponkus and Lynch, 1989). The cryobehavior of liposomes prepared from plasma membrane lipids extracted from cold acclimated and

nonacclimated rye leaves during freeze-induced osmotic contraction was analyzed and it was shown that the differential cryostability of the plasma membrane of cold acclimated and nonacclimated plant cells was indeed determined by alterations in the composition of the lipid bilayer. In liposomes made from plasma membranes of nonacclimated cells, numerous vesicles were subdued from the liposome bilayer and sequestered into the liposome interior during freeze-induced osmotic contraction. These liposomes lysed during subsequent osmotic expansion upon thawing. Under similar conditions, liposomes prepared from membrane lipids of cold acclimated cells did not exhibit such behavior. Instead, osmotic contraction resulted in the formation of either exocytotic extrusions or vesicles that remained contiguous with the parent bilayer. During subsequent thawing and osmotic expansion, the extruded vesicles were drawn into the plane of the parent liposome bilayer and lysis was avoided.

The specific lipid composition responsible for the cryostability of cold acclimated plant cells has been studied using pH-induced protoplast x liposome fusion techniques (Steponkus *et al.*, 1988). It was found that the freezing tolerance of nonacclimated protoplasts at temperatures ranging from $0^{o}C$ to $-5^{o}C$ could be dramatically increased (from 50% survival to 100% survival) by fusing nonacclimated protoplasts with liposomes composed of mono-unsaturated or diunsaturated molecules of phosphotidylcholine. At temperature over the range of $0^{o}C$ to $-5^{o}C$, the principal form of injury in nonacclimated protoplasts was expansion-induced lysis, which was probably the consequence of endocytotic vesiculation in the plasma membrane during osmotic contraction. Remarkably, when the nonacclimated protoplasts were fused with mono- or diunsaturated species of phosphotidylcholine, they formed exocytotic extrusions during freeze-induced osmotic contraction and the incidence of osmotic expansion induced lysis diminished. However, it did not appear that mono- or

diunsaturated phosphotidylcholine were responsible for the functional and morphological behavior of cold acclimated protoplasts at temperatures of around -10°C. Nevertheless, the changes in plasma membrane lipid composition appear to be important for one facet of the cold acclimation process.

B. Protein Changes Associated with Cold Acclimation

Two lines of evidence suggest that alteration in protein composition of plants may contribute to the freezing tolerance developed during cold acclimation. The evidence consists of repeated observations that a number of enzymes show shifts in isozyme composition during cold acclimation, and numerous studies have shown both quantitative and qualitative differences in the protein composition between nonacclimated and cold acclimated plant tissues (see Levitt, 1980; Thomashow, 1990; Guy, 1990).

Studies of a number of enzymes from cold-acclimated plants have demonstrated changes in activity, stability, and isozyme variation when compared to nonacclimated ones. One of the best-characterized enzyme relative to cold acclimation is ribulose bisphosphate caboxylase/oxygenase (Rubisco) from winter rye, one of the most freezing-tolerant cereals. It was found that cold acclimated and nonacclimated rye have different Rubisco isozymes (Huner and Macdowall, 1976, 1978, 1979). The enzyme isolated from cold acclimated plants was not only more stable to denaturants and freezing storage, but also had a lower Km for CO_2 at temperatures below 5°C. Similar studies using the enzyme isolated from freezing-sensitive and tolerant potato species also demonstrated structural differences that paralleled the variation in freezing tolerance much in the same way the enzyme did from acclimated and nonacclimated rye (Huner *et al.*, 1981). When the Rubisco isozymes from cold acclimated and nonacclimated cabbage were isolated (Shomer-Ilan and Waisel, 1975), their amino acid

compositions were found to be significantly different, which implied an involvement of differential gene expression, as described later.

A number of other examples of cold-acclimation-associated alteration in isozyme profiles has been documented. These include peroxidase (McCown *et al.*, 1969), invertase (Roberts, 1974), ATPase, esterase, acid phosphatase, leucine aminopeptidase (Krasnuk *et al.*, 1976), and a number of dehydrogenase associated with the respiratory pathway (Hall *et al.*, 1970). It has been suggested in many cases that the "acclimated" isozymes might be better "suited" to a low temperature environment. For example, a study of glutathione reductase from spinach has demonstrated that the isozyme isolated from acclimated plants was less sensitive to freeze/thaw inactivation than its nonacclimated counterpart (Guy and Carter, 1984).

An indication that *de novo* protein synthesis is required for the induction of freezing resistance comes from studies using the protein synthesis inhibitor cycloheximide. It has been found in a number of plants, including winter wheat, winter rape, and potato species that application of cycloheximide could inhibit the development of freezing tolerance by cold acclimation (Trunova and Zvereva, 1977; Kacperska-Palacz *et al.*, 1977; Chen *et al.*, 1983). In addition, a general observation has been made that the soluble protein content of plants increases during cold acclimation (see Levitt, 1980; Sakai and Larcher, 1987). Examples include studies as long as 40 years ago in the black locust tree (Siminovitch and Briggs, 1949) where the protein content was found to increase during cold acclimation. In a more recent study, the content of protein of acclimated wheat was estimated to be 300% greater than that of the nonacclimated plants (Trunova, 1982). Alterations have also been reported in membrane protein composition during cold acclimation in orchard grass and winter rye (Yoshida and Uemura 1984, Uemura and Yoshida, 1984), where the plasma membrane

protein profiles were found to be different between cold-acclimated and nonacclimated plants. Moreover, the polypeptide composition of tissues from a variety of cold -acclimated and nonacclimated plants have been shown to be different when examined by electrophoresis and *in vivo* radiolabeling (see Thomashow 1990; Guy, 1990). Studies of cold-acclimation associated changes in polypeptide composition and protein from a variety of plant species can be summarized as follows:

(1) Cold acclimation is associated with both the appearance of new polypepetides and increase and/or decreases in others.

(2) Although a number of changes occur, the overall patterns of protein synthesis in cold-acclimated and nonacclimated plants appears to be quite similar, which is in contrast with other stress responses like that of heat shock or anaerobiosis. Another difference between the response of protein synthesis to cold stress and other stresses is that the response is relatively slower to cold in comparison to other stresses.

(3) The newly synthesized proteins, also referred to as COR (cold-regulated) proteins (Thomashow, 1990), range from large (200 kDa) to small (< 10 kDa) and from basic (pI > 8) to acidic (pI < 4). They can be soluble or membrane-bound proteins. There is not a simple pattern which is universal between species.

(4) The appearance of most, if not all, of the COR polypeptides generally coincides with the onset of freezing tolerance; their synthesis continues as long as the plants are kept at cold-acclimating temperatures, and their synthesis declines quickly during deacclimation.

In most cases, the functional significance of changes in isozyme or other protein composition during cold acclimation is not known. Some of these changes may contribute indirectly to the overall fitness of the plant for low temperarue survival, which in turn could increase plant freeze tolerance. For example, new isozymes may be necessary to accomplish the necessity to shift metabolic pathways at low

temperature, or replace cold labile isozymes so that the plant can continue to operate the pathway at low temperature. Other changes may contribute directly to the freezing tolerance of acclimated plants. For example, Volger and Heber have reported that some proteins (10-20 kDa) which accumulate in acclimated spinach leaves have cryoprotective activity in an *in vitro* assay (Volger and Heber, 1975), as do sucrose, trehalose, proline and other small molecules (Crowe and Crowe, 1986; Carpenter and Crowe, 1988; Caffrey *et al.*, 1988). The activity of these cryoprotective proteins, measured as the ability to protect photophosphorylation of the thylakoid membrane against freezing damage, was found to be much higher (more than 10,000 times higher on a molar basis) than that of small molecular weight cryoprotectants. Similar cryoprotective proteins have also been reported in cold acclimated cabbage (Hincha *et al.*, 1989; Hincha and Schmitt, 1990). The cabbage proteins enriched of a polypeptide of about 28 kDa, was found to be 20,000 to 40,000 times as effective as sucrose in preventing freeze-thaw induced rupture of isolated thylakoid membranes. Both the spinach and cabbage proteins are very hydrophilic and stable to conditions that are usually detrimental to proteins, such as extreme pH, and extremely high temperature (95^{o}C to boiling). However, these "cryoprotective" proteins have not been isolated to purity and whether these proteins have important cryoprotective functions *in vivo* and contribute significantly to freezing tolerance of the plants also remain to be determined.

III. Genetics of Cold Acclimation

A. Inheritance of freezing tolerance

The inheritance of freezing tolerance was studied first by Nilsson-Ehle (1912) who crossed two winter wheat varieties intermediate in freezing tolerance and found

transgressive segregation (progeny segregate outside the parental boundaries) for the trait of freezing tolerance. He concluded that freezing tolerance was a quantitatively inherited trait controlled by multiple genes. Subsequent studies have confirmed this conclusion (Hayes and Admodt, 1927; Rudolph and Nienstaedt, 1962; Norell *et al.*, 1986; Limin and Fowler, 1988). In wheat, barley and oat, several studies have reported evidence that freezing tolerance is controlled by partially dominant and/or recessive genes (Rhode and Pulham, 1960; Jenkins, 1969; Gullord *et al.*, 1975; Sutka, 1981; Orlyuk, 1985). The complexity of genetics for freezing tolerance in plants is further revealed by the detailed cytogenetic studies of wheat using monosomic and substitution analysis. Eleven out of 21 chromosomes of hexaploid wheat have been suggested to be involved in freezing tolerance although chromosome 5A and 5D appear to carry the major effects (see Roberts, 1986; Thomashow, 1990). The repeated verification of the quantitative nature and the complexity of the genetics of plant freezing tolerance is not unexpected given the fact that morphology, physiology, developmental processes, and environmental interactions all influence the ability of a plant to survive in winter.

B. Changes in gene expression during cold acclimation

Over the last two decades, it has become apparent that plants respond to adverse environmental stresses (anaerobiosis, heat shock, water deficit, salt stress, etc.) through alterations in gene expression (Key and Kosuye, 1984; Sachs and Ho, 1986). In general, when the environmental conditions become unfavorable for optimal growth and development of the plant, new stress-related genes are induced and their products accumulate. Although the functional identity for most stress-related genes remains undetermined, it is certain that at least some of them function to enhance the plant's survival (Sachs and Ho, 1986). Prior to advances in the understanding of gene

expression and regulation in anaerobic and heat shock stresses, the two most extensively studied cases, a role for changes in gene expression in cold acclimation and freezing tolerance was suggested. As early as 1970 Weiser proposed that altered gene expression and the synthesis of new proteins was necessary for the induction of maximum freezing tolerance in temperate perennials (Weiser, 1970). As previously described, studies in protein biochemistry have been generally supportive of Weiser's hypothesis, but the specific changes in gene expression responsible for freezing tolerance is still unknown.

It is now clear that changes in gene expression occur during cold acclimation at both the transcriptional and post transcriptional levels (Thomashow, 1990). The first evidence along this these lines came from the work of Guy and colleagues (Guy *et al.*, 1985). By comparing *in vitro* translation products of poly(A^+) RNA isolated from control and cold acclimated spinach plants, these authors found that mRNAs encoding 180 and 82 kDa polypeptides accumulated in cold acclimated plants. Upon a longer period of cold acclimation, additional RNA species encoding different polypeptides accumulated in acclimated plants. It was also clear from this work that the overall RNA populations in nonacclimated and cold acclimated plants did not differ dramatically. This result is in agreement with the results from the studies of protein synthesis studies discussed before. Cold acclimation associated changes in mRNA populations have since been reported in a number of plant species. The general observation is similar to the one reported by Guy *et al.*, although the specific RNAs altered by cold temperature are different from species to species. For example, in cold acclimated rapeseed (*Brassica napus*), RNAs encoding nine polypeptides ranging from 25 to 80 kDa accumulated (Meza-Basso *et al.*, 1986). Translatable RNA for seven polypeptides (11 to 95 kDa) in alfalfa (*Medicago falcata* cv Anik) were found to increase during cold acclimation (Mohapatra *et al.*, 1987). Levels of RNA for 160 and

47 kDa polypeptides were found to be elevated in cold acclimated *Arabidopsis thaliana* (Gilmour *et al.*, 1988).

Since the late 1980s, cDNA clones representing *cor* (cold regulated) genes have been isolated from several plant species including alfalfa, barley, wheat, rapeseed, and *Arabidopsis* (Mohapatra *et al.*, 1988, 1989; Kurkela and Franck, 1990; Hajela *et al.*, 1990; Cattivelli and Bartels, 1990; Houde *et al.*, 1991; Singh *et al.*, 1991). In all these cases, the *cor* cDNA clones were isolated using the technique of differential screening which has been almost a standard approach in the isolation of stress -responsive genes (Key and Kosuge, 1984). Many *cor* genes have been suggested to be members of small gene families (Mohapatra *et al.*, 1989; Kurkela and Franck, 1990; Houde *et al.*, 1991). Northern analysis of *cor* gene expression showed that the steady state level of *cor* gene transcripts accumulated as quickly as 12 hours after cold induction in *Arabidopsis* (Hajela *et al.*, 1990) or as slowly as 2 to 3 days in alfalfa (Mohapatra *et al.*, 1988). The level of these transcripts usually increased to 10 fold or higher in cold acclimated plants compared to the nonacclimated level, and returned to to lower level in a few hours upon deacclimation. Nuclear run-on transcription analysis indicated that of four *Arabidopsis cor* genes, three were regulated primarily at the post-transcriptional level while the other one was controlled largely at the transcriptional level (Hajela *et al.*, 1990).

C. Characterization of *cor* genes and products

No functional identity of any *cor* gene has been determined. However, a correlation between the plant freezing tolerance and the *cor* gene expression has been reported in alfalfa (Mohapatra *et al.*, 1989). Alfalfa cultivars Anik (*Medicag falcata*), Iroquois (*M. sativa*), Algonquin (*M.media*), and Trek (*M. sativa*) have different degrees of freezing tolerance: when cold acclimated, they have LT_{50} (the temperature

resulting in 50% mortality) values of -14.6, -11.8, -11.5, and -9.7°C, respectively. The relative level of expression of three *cor* genes, represented by cDNA clones pSM7844, pSM2358, and pSM2201 were evaluated in the alfalfa cultivars by Northern blot analysis. A positive correlation (with correlation coefficients larger than 0.9) was observed between the RNA levels for the genes represented by the three cDNA clones and the degree of freezing-tolerance in four alfalfa cultivars. It is not clear whether this type of correlation exists in other plant species.

Many studies on the characterization of *cor* genes have been directed at determining whether their expression is regulated by the plant hormone abscisic acid (ABA) and by drought stress. The reason that ABA is of interest is that exogenous application of ABA has been shown to increase freezing tolerance in whole plants and plant cell cultures in the absence of cold treatment (Irving, 1969; Rikin *et al.*, 1975; Chen and Gusta, 1983; Chen *et al.*, 1983; Orr *et al.*, 1986; Reaney and Gusta, 1987; Mohapatra *et al.*, 1988; Lang *et al.*, 1989). Moreover, those species that increase in freezing tolerance in response to cold (eg. *Medicago sativa, Brassica inermis, Daucus carota, and Triticum aestivum*) also become more freezing tolerant in response to ABA while those species that do not frost harden in response to cold (eg. *Datura innoxia, Catharanthus roseus, Glycine max, Vicia hajastana*, and cell lines of *Triticle*) do not accliamte in response to ABA. Therefore, an important question raised by the ABA experiments is whether the expression of *cor* genes is also modulated by ABA. This seems to be the case for some *cor* genes but not for others. For example, all of the *Arabidopsis cor* genes whose cDNA clones have been isolated are responsive to ABA (Hajela *et al.*, 1990; Kurkela and Frank, 1990); on other hand, of four alfalfa *cor* cDNA clones (Mohapatra et al., 1988, 1989), only one, (pSM1409, the relationship of its expression with the degree of freezing tolerance is not known) was found to be responsive to ABA (Mohapatra *et al.*, 1988) but only in the relatively frost-resistant

cultivars; other alfalfa *cor* genes whose expression was strongly correlated with the freezing tolerance of the alfalfa cultivars described previously, however, were not responsive to ABA (Mohapatra *et al.*, 1989). Cold-regulation and ABA-regulation of the *cor* genes have been suggested to be mediated through independent control mechanisms by the fact that ABA-regulated expression of *Arabidopsis cor* genes were dramatically impaired in an ABA-insensitive *Arabidopsis* mutant, *abi1*, while the cold -regulated expression of these genes was unaffected in the *abi1* plants (Gilmour and Thomashow, 1992).

To determine whether *cor* gene expression is affected by water deficit is of interest for at least three reasons. First, it is well established that drought stress results in the accumulation of ABA (see Levitt, 1980), and the increased ABA content of plant during cold acclimation has also been reported (Chen *et al.*, 1983). Therefore, *cor* genes which are responsive to ABA might as well be responsive to drought stress. Second, several studies have found that drought stress could induce freezing tolerance at normal growth temperature for some plants, such as cabbage, wheat, and rye (Cox and Levitt, 1976; Cloutier and Siminovitch, 1982; Siminovitch and Cloutier, 1982,1983). Finally, severe dehydration of the plant cell is one of the consequences resulting from extracellular freezing. As a consequence of the similarity between freezing and drought stress at the cellular level, plants may have developed similar responses to both forms of the stress, that involve common adaptive mechanisms. Thus, it is reasonable to hypothesize that genes with roles in drought stress might also have functions in freezing tolerance, and that a set of genes might be regulated by both kinds of stress. There have been limited studies in alfalfa and *Arabidopsis* on the expression of *cor* genes in response to drought stress. In alfalfa, it was found that the expression of one *cor* gene (pSM1409; the one responsive to ABA), was indeed increased 10 fold in water stressed plants (Mohapatra *et al.*, 1988). In contrast, three

other alfalfa *cor* genes which were not responsive to ABA were apparently not regulated by water stress (Mohapatra *et al.*, 1989). On the other hand, all *Arabidopsis* cor genes whose cDNA clones had been isolated are stimulated by water stress (Hajela *et al.*, 1990).

Although it is clear that reproducible changes in gene expression occur during cold acclimation, the roles of these genes in cold acclimation are unknown. There is no report on the detailed characterization of any COR polypeptide, nor is their cellular localization known. Most importantly, there is no direct evidence for or against the hypothesis that COR polypeptides have significant roles in freezing tolerance as was first proposed about two decades ago (Weiser, 1970). Detailed studies on *cor* genes and COR polypeptides should provide answers to the above questions.

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Chapter 2

ARABIDOPSIS cor GENES ENCODING BOILING-STABLE POLYPEPTIDES

SUMMARY

Changes in gene expression during cold acclimation of *Arabidopsis* were studied by analyzing the *in vitro* translation products of RNAs isolated from control and cold -acclimated plants. Transcripts encoding polypeptides with molecular weights of approximately 160, 47, 24, and 15 kDa were found to accumulate in cold-acclimated plants. These polypeptides, referred to as COR160, COR47, COR24, and COR15, respectively, share the unusual biochemical property of remaining soluble upon boiling in aqueous solution. The accumulation of transcripts encoding "boiling-stable" polypeptides was also detected in cold-treated spinach and potato. The *Arabidopsis* genes encoding COR160, COR47, and COR15 were shown to be represented by cDNA clones pHH28, pHH7.2, and LCT10, respectively. Drought-stressed *Arabidopsis* plants were found to accumulate transcripts for COR160, COR47, COR24, and COR15 as well as a number of drought-specific transcripts that also encode boiling-stable polypeptides.

INTRODUCTION

Cold acclimation is a complex response involving a variety of physical and biochemical changes (Levitt, 1980; Sakai and Larcher, 1987). One of the hallmarks of cold acclimation is increased freezing tolerance; in many species of plants, a period of exposure to low nonfreezing temperatures results in an increased tolerance to freezing temperatures (Levitt, 1980; Sakai and Larcher, 1987). 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 (Levitt, 1980; Sakai and Larcher, 1987; Steponkus and Lynch, 1989). 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 (Steponkus and Lynch, 1989; Steponkus *et al.*, 1988). In addition, there is evidence that proline and many simple sugars (Carpenter and Crowe, 1988; Santarius, 1973; Strauss and Hauser, 1986), as well as certain soluble polypeptides from spinach (Volger and Heber, 1975), have cryoprotective effects *in vitro*. Whether these molecules contribute significantly to freezing tolerance *in vivo*, however, remains to be determined.

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 (Weiser, 1970), that cold acclimation involves changes in gene expression. Indeed, recent studies with a variety of plant species including *Arabidopsis* have

demonstrated that changes in gene expression occur during cold acclimation (see Guy, 1990; and Thomashow, 1990). Very little is known, however, about the nature of the *cor* (cold-regulated) genes. Are any of the polypeptides encoded by *cor* genes related at the structural or functional level? Are *cor* genes involved in freezing tolerance or other aspects of low temperature survival? Are *cor* genes responsive to environmental signals other than low temperature? To address these issues, I have initiated a characterization of *cor* genes and their products in *Arabidopsis thaliana*. I have chosen *Arabidopsis thaliana* as a model system as it has the ability to cold acclimate (Gilmour *et al.*, 1988) and it is well suited for genetic and molecular genetic studies (Meyerowitz and Pruit, 1985; Estelle and Somerville, 1986).

MATERIALS AND METHODS

Plant Material

Arabidopsis thaliana L.(Heyn) ecotype Landsberg or Columbia was grown in controlled environment growth chambers at 21^{o}C under constant illumination from cool white fluorescent lights. Light intensity was approximately 150 uE $m^{-2}s^{-1}$ near soil level, and the relative humidity was about 80%. *Arabidopsis* plants (approximately two weeks old) were cold acclimated by transferring them to chambers set at 5^{o}C for 3 days with continuous light at an intensity of approximately 50 uE m^{-2} s^{-1}. Drought stress was applied to plants grown at 21^{o}C by withholding water until they became visibly wilted (approximately 8 days). Seedlings of spinach (*Spinacia oleracea* L.) and potato species (*Solanum tuberosum, S. acaule, S. commersonii*) were grown under similar conditions to that of *Arabidopsis*, and were cold acclimated at 4^{o}C for 4 days and 7 days, respectively.

For RNA extraction, *Arabidopsis* rosette leaves, and spinach and potato leaves, were excised, ground in liquid N_2 with a mortar and pestle and either used directly or stored at -80°C prior to use.

Total and Poly(A^+) RNA Isolation

Total RNA and poly(A^+) RNA were prepared from plants essentially as described (Gilmour *et al.*, 1988). Frozen pulverized plant material was collected and extracted in a buffer (4 ml per gram tissue) containing 100 mM Tris-HCl (pH 7.6), 50 mM EGTA, 100 mM NaCl, 1% (w/v) SDS, 10 mM DTT, 6% (w/v) *p*-aminosalicylic acid (sodium salt) (Kodak), and 1% (w/v) tri-isopropylnaphthalene-sulfonic acid (sodium salt) (Sigma), and an equal volume of buffer-saturated phenol/chloroform/isoamyl alcohol (25/24/1, v/v/v). After centrifugation (10,000 g, 10 min) to separate the phases, the aqueous phase was extracted again with phenol/chloroform/isaomyl alcohol, and the nucleic acids were ethanol precipitated. All the solutions used after this point were made free of RNase by DEPC treatment (Sambrook *et al.*, 1989). The pellets from ethanol precipitation were resuspended in water, then precipitated with 2 M LiCl, followed by ethanol precipitation. The pellets were dissolved in water and stored at -80°C. Poly(A^+) RNA was further isolated from total RNA using poly(U) Sepharose chromatography according to Cashmore (Cashmore, 1982).

***In Vitro* Translation**

Poly(A^+) RNA or total RNA was translated *in vitro* using a rabbit reticulocyte lysate system (Promega) with [^{35}S]methionine (10^3 Ci/mmole) as a radiolabel according to the manufacturer's instruction. Typically, 1 ug of poly(A^+) RNA or 5 to

10 ug of total RNA was translated in a 50 ul reaction containing 35ul lysate and about 50 uCi [^{35}S]methionine at 30^{o}C for 1 h.

Polyacrylamide Gel Electrophoresis

SDS-PAGE gels were run according to the method of Laemmli (Laemmli, 1970). For most of the experiments, equal volumes of the *in vitro* translation reaction were analyzed, generally 2 to 4 ul for SDS-PAGE. Gels were stained with Coomassie brilliant blue to visualize the molecular weight standards, dried, and autoradiographed for up to 1 week. In some experiments, the gel was soaked in 1 M sodium salicylate prior to drying, then fluorographed for 1 to 3 days. Kodak X-Omat AR5 x-ray film was used for both the autoradiography and fluorography.

Detection of Boiling-Stable COR Polypeptides

Poly(A^{+}) RNA or total RNA was translated *in vitro*. The translation reactions 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 microcentrifuge (15 min). Polypeptides that remained soluble were precipitated with 5 volumes of acetone and collected by centrifugation in a microcentrifuge (15 min). The pelleted material was analyzed by SDS-PAGE gels as described above.

cDNA Clones of *cor* Genes

cDNA clones pHH28, pHH7.2 and pHH67 were previously described (Hajela, *et al.*, 1990). pLCT10A and pLCT10B are cDNA clones homologous to pHH67, but were isolated from a different cDNA library prepared as follows. Double-stranded cDNA was synthesized from poly(A^{+}) RNA isolated from 3-day-cold-acclimated

Arabidopsis (Columbia) essentially as described by Gubler and Hoffman (Gubler and Hoffman, 1983). *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 $5x10^6$ primary recombinants were obtained. The library was amplified once and stored at -70°C 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 (Sambrook *et al.*, 1989).

The cDNA library was screened to isolate clones homologous to *cor* cDNA clone pHH71.1, a homologue of pHH67 (Hajela *et al.*, 1990). Plaque lifts were prepared on Nytran membranes (Schleicher and Schuell) using standard methodologies (Ausubel *et al.*, 1987; Sambrook *et al.*, 1989). The cDNA insert from pHH71.1 was ^{32}P-labeled by random priming (Feinberg and Vogelstein, 1983). Hybridization was at 60°C in buffer containing 6 X SSC (1 X 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 X SSC, 0.5% (w/v) SDS at 60°C. Phage displaying strong homology to the pHH71.1 probe were plaque purified and the cDNA inserts "subcloned" in pBluescript SK(-) by a temperature induced "phagemid rescue" procedure according to the supplier's instructions (Stratagene). The resulting plasmids was analyzed by Southern hybridization using the pHH71.1 probe. One clone chosen for further analysis was pLCT10A. The orientation of the *Eco*RI insert in pLCT10A was reversed using standard recombinant DNA methods (Sambrook *et al.*, 1989) to give pLCT10B.

Hybrid-Arrest *in Vitro* Translation

Hybrid-arrest translations were done as described (Jagus, 1987) using poly(A^+) RNA and single-stranded DNA. Single-stranded DNA antisense to the mRNAs were prepared from cDNA clones pHH28, pHH7.2, pLCT10A, and the cloning vector pBluescript SK^- by phage rescue method as described by Vieira and Messing (Vieira and Messing, 1987). To initiate a hybrid-arrest translation reaction, 1 ug poly(A^+) RNA isolated from 3-day-cold-acclimated plants, 0.5 ug single-stranded DNA, and 10 ug tRNA were precipitated together with ethanol. The pellet was resuspended in 3 ul water, boiled in a water bath for 30 seconds, snap-frozen in liquid N_2, thawed on ice, and mixed with 23 ul of hybridization solution (hybrid solution is 80% deionized formamide, 400 mM NaCl, 10 mM Pipes-NaOH pH 6.4, 8 mM EDTA). The reaction was incubated at 37°C for 10 hours, the nucleic acids precipitated with ethanol, the pellet resuspended, translated *in vitro*, and the translation products analyzed by SDS -PAGE.

***In Vitro* Transcription/Translation**

pLCT10A and pLCT10B were linearized by digestion with *Bam*HI and the inserts transcribed *in vitro* with T7 RNA polymerase (Promega) using the T7 promoter carried on the pBluescript vector. The resulting transcripts were extracted with phenol:chloroform:isomayl alcohol (25:24:1), and precipitated with ethanol. Transcription products were translated *in vitro* using the rabbit reticulocyte lysate system (Promega) containing [^{35}S]methionine as described. Boiling-stable polypeptides were prepared, and the radioactive polypeptides were fractionated by SDS-PAGE and visualized by autoradiography.

RESULTS

Cold Acclimation is Associated with Accumulation of Transcripts Encoding Boiling-Stable Polypeptides.

Cold acclimation of *Arabidopsis* results in the accumulation of transcripts encoding polypeptides of 160 kDa, 47 kDa, 24 kDa and 15 kDa (Gilmour et al., 1989; Lin unpublished); these polypeptides are referred to as COR160, COR47, COR47, and COR15, respectively. As a first step towards determinng the functions of these COR polypeptides, I initiated a characterization of their physical properties. I found that all four 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 were first boiled, centrifuged to remove the boiling-insoluble material, and then fractionated by SDS-PAGE gels (Figure 2.1). 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, all four of the major COR polypeptides remained soluble. In addition, transcripts encoding boiling-stable polypeptides of 18, 20, and 21 kDa appeared to increase in the acclimated plants.

Accumulation of transcripts encoding boiling-stable polypeptides at low temperatures is not unique to *Arabidopsis*; it also occurs in at least spinach and potato. In particular, boiling-stable polypeptides with the apparent molecular weight of approximately 24 and 12 kDa synthesized by RNAs isolated from cold-acclimated but not nonacclimated spinach plants (Figure 2.2). Similarly, cold-treatment resulted in the accumulation of RNA species encoding boiling-stable polypeptides in three *Solanum* species (Figure 2.2). For example, the boiling-stable polypeptides specific to cold

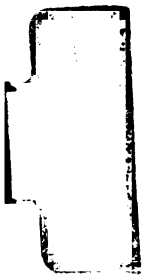

Figure 2.1. Accumulation of transcripts encoding boiling-stable polypeptides in cold acclimated *Arabidopsis*. Poly(A)$^+$ RNA isolated from cold acclimated (AC) and nonacclimated (NA) *Arabidopsis* was translated *in vitro* and the polypeptide products were either fractionated directly by SDS-PAGE (TOTAL) 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 days.

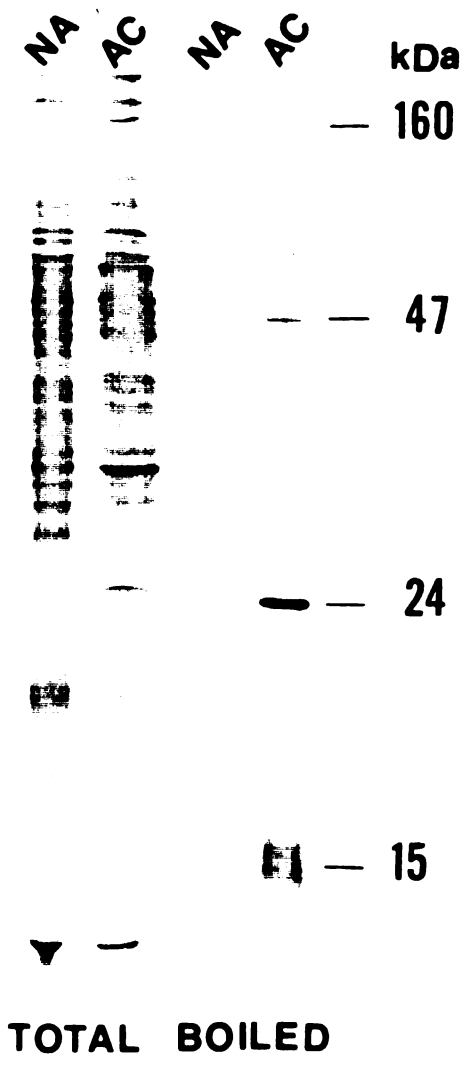

Figure 2.1

Figure 2.2 Analysis of boiling-stable polypeptides synthesized by total RNAs isolated from nonacclimated (NA) and cold-acclimated (AC) spinach (spin.), and *Solanum* species: *S. acaule* (S.a.), *S. commersonii* (S.c.), and *S. tuberosum* (S.t.). Asterisks indicate the boiling-stable COR polypeptides. MW indicates the molecular weight ($x10^{-3}$) of the standards.

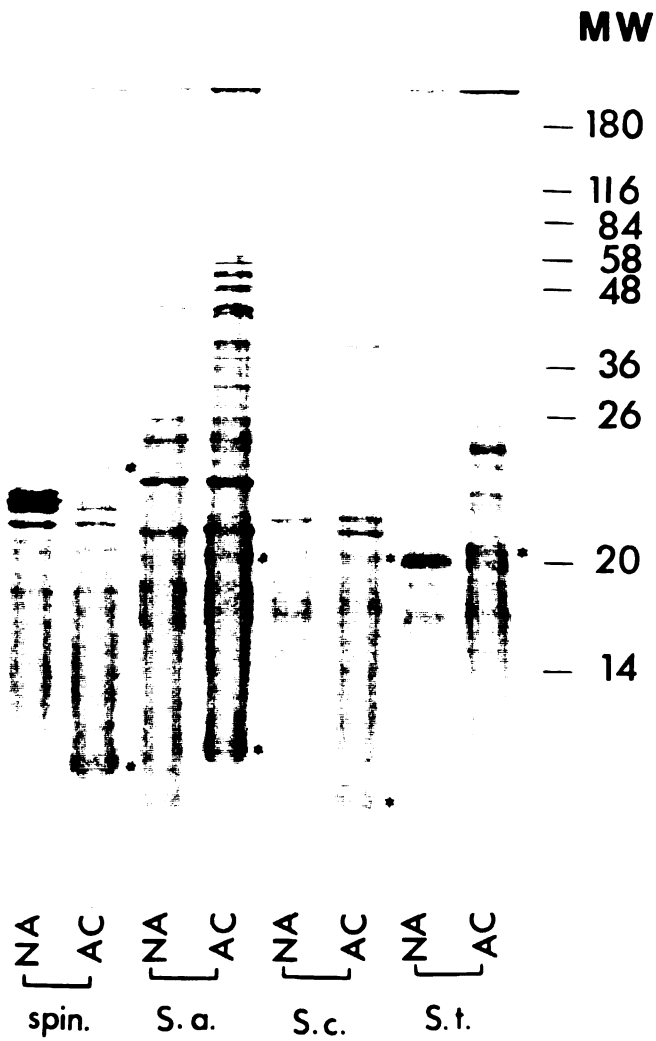

Figure 2.2

-treated plants include those of approximately 20 and 12.2 kDa in *S. acaule*, 20 and 11.8 kDa in *S. commersonii*, and 20.2 kDa in *S. tuberosum* (Figure 2.2).

Identification of cDNA Clones Encoding *Arabidopsis* Boiling-Stable Polypeptides.

A previous study resulted in the isolation of four *Arabidopsis cor* genes (Hajela *et al.*, 1990). It was of interest to determine whether any of these genes encoded a boiling-stable COR polypeptide. Hybrid-arrest *in vitro* translation experiments indicated that at least three of them did. In particular, pHH28, pHH7.2, and pLCT10A inhibited the translation of COR160, COR47, and COR15, respectively (Figure 2.3). That the pLCT10A insert encoded COR15 was confirmed by an *in vitro* transcription/translation experiment (Figure 2.4). When pLCT10B, which has the same insert as pCTL10A but in the opposite orientation, was transcribed *in vitro* using T7 polymerase (the vector carries a T7 promoter) and translated *in vitro*, a boiling -stable polypeptide of 15 kDa was synthesized. This polypeptide was not synthesized in the *in vitro* transcription/translation reactions using pLCT10A. The gene encoding COR15 was designated *cor15*.

Drought Stress in *Arabidopsis* is Associated with Accumulation of mRNAs Encoding Boiling-Stable Polypeptides

Freezing stress, to a large extent, is a dehydration stress. Therefore, mechanisms enabling plants to tolerate freezing stress might have components in common with those for dehydration tolerance. Specifically, similar changes in gene expression might occur in both low temperature and drought stresses. This was demonstrated to be the case by analyzing the *in vitro* translation products of mRNAs isolated from control, cold-acclimated, and drought stressed plants (Figure 2.5). It is clear that mRNAs encoding COR160, COR47, COR24, and COR15 accumulated in

Figure 2.3. Hybrid-arrest *in vitro* translation experiments indicating that *Arabidopsis* cDNA clone pHH28, pHH7.2 and pLCT10A (indicated as pLCT10) represent *cor* genes encoding boiling-stable COR polypeptides. Poly(A+) RNA isolated from nonacclimated (NA) and cold acclimated (AC) plants was either translated *in vitro* without hybrid-arrest (NA and AC), or after hybrid-arrest with single stranded DNA of pHH28, pHH7.2, pLCT10A, and cloning vector pBluescript (pBS). The boiling-stable products of the translation were fractionated with a 15% SDS-PAGE, and the autoradiograph shown.

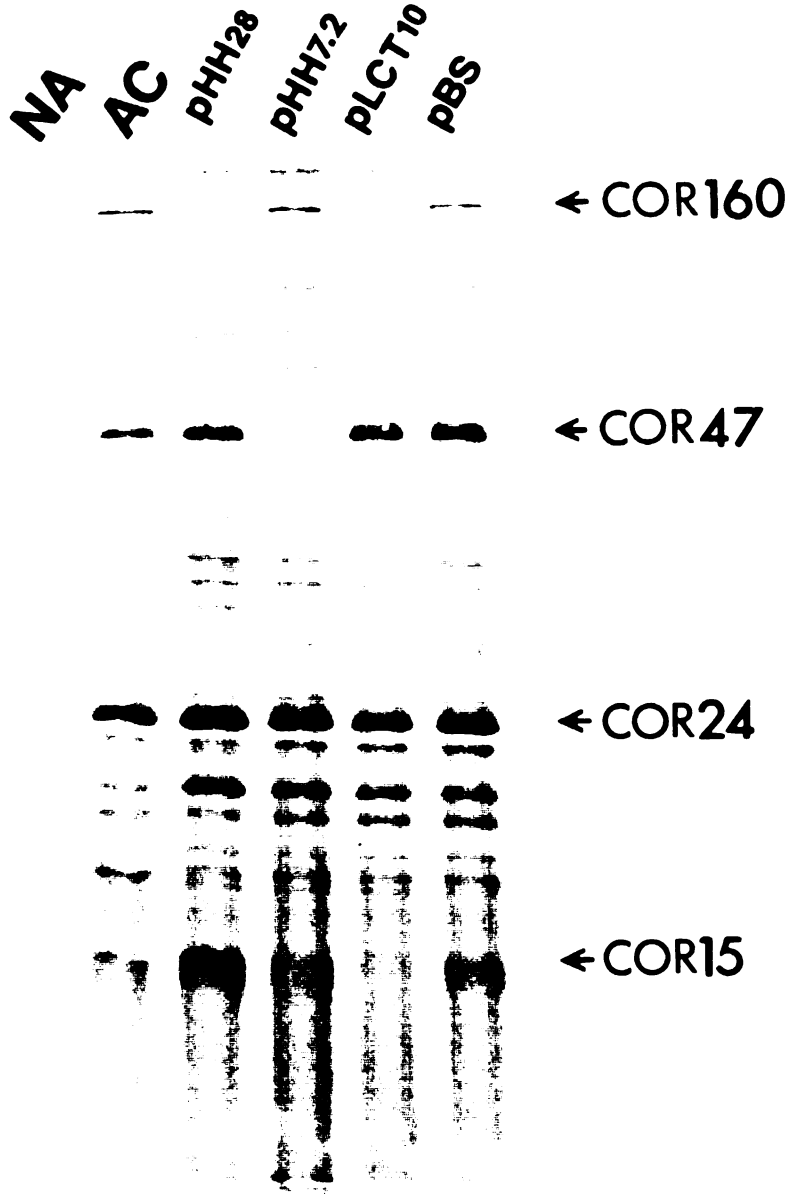

Figure 2.3

Figure 2.4 cDNA clone pLCT10B encodes a 15 kDa boiling-stable polypeptide comigrating with COR15 in SDS-PAGE. Lanes 1 and 2 show the boiling-stable polypeptides synthesized by *in vitro* translation of poly(A^+) RNAs isolated from nonacclimated (lane 1) and cold-acclimated (lane 2) plants. Lanes 3 and 4 show the *in vitro* transcription/translation products of pLCT10B (lane 3) and pLCT10A (lane 4). MW indicats the molecular weight (x 10^{-3}) of the major COR polepeptides.

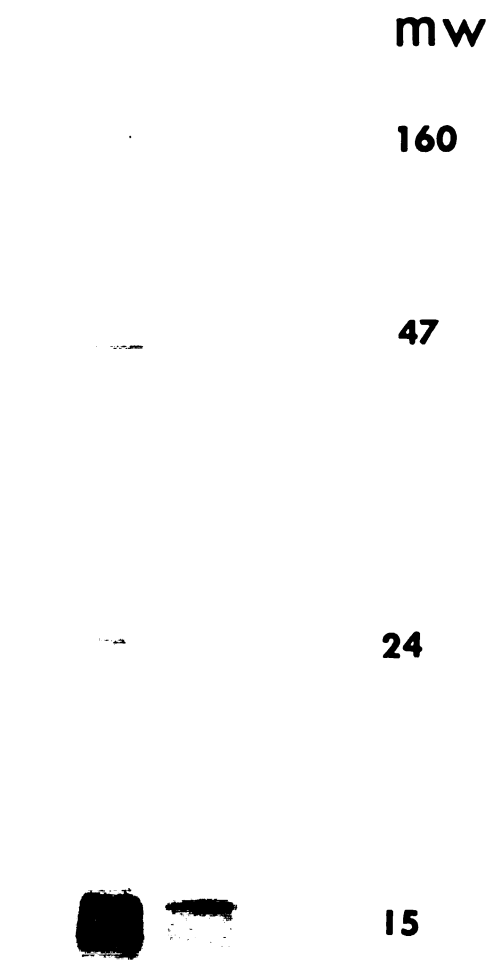

Figure 2.4

Figure 2.5. Accumulation of transcripts encoding COR polypeptides and other boiling-stable polypeptides in drought stressed *Arabidopsis*. Lanes 1 and 2 show the total *in vitro* translation products synthesized from poly(A+) RNA isolated from control plants (lane 1), and drought-treated plants (lane 2). Lanes 3 and 4 show the boiling soluble fractions of the *in vitro* translation products synthesized from poly(A+) RNA isolated from drought-treated plants (lane 3), and cold-treated plants (lanes 4). The asterisks indicate the boiling-stable COR polypeptides, and the arrow heads indicate the boiling-stable polypeptides specific to drought.

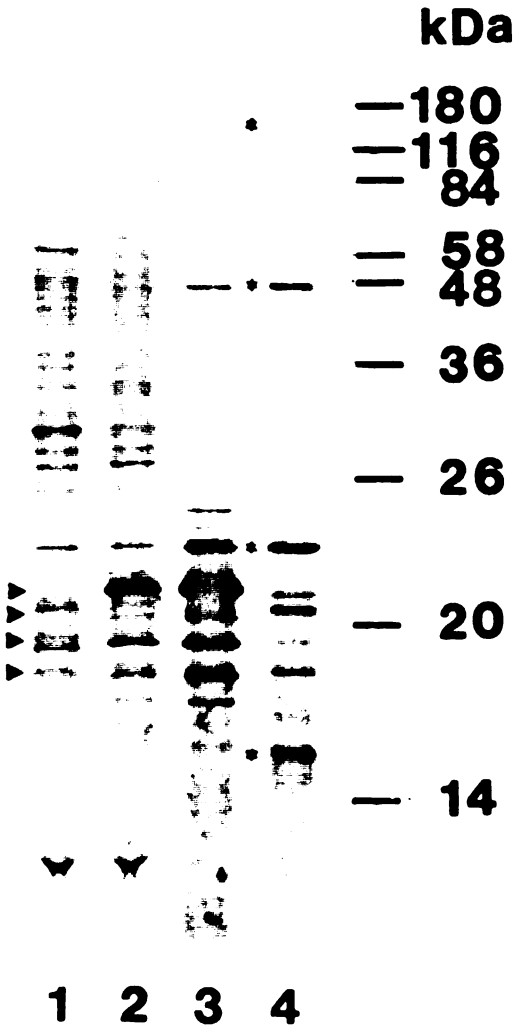

Figure 2.5

both cold-treated and drought-stressed plants (Figure 2.5, lanes 4 and 3), although the level of these *cor* transcripts seemed to be lower in drought-stressed plants than in the cold-treated plants. Besides the accumulation of the *cor* gene products, drought -stress resulted in accumulation of transcripts encoding an additional set of boiling -stable polypeptides of approximately 22, 20, 19 and 18 kDa (Figure 2.5, lane 3), which were not found in either control or cold-treated plants (Figure 2.5, lane 1, and 4).

DISCUSSION

The data presented indicate that several of the *cor* transcripts from *Arabidopsis* encode polypeptides share the unusual biochemical property of remaining soluble upon boiling in aqueous solution. Four *Arabidopsis cor* genes have been isolated in a previous study by differential screenings of a cDNA libaray (Hajela *et al.*, 1990). Three of these *cor* genes, represented by cDNA clones pHH28, pHH7.2, and pLCT10A, are shown here to encode the boiling-stable COR polypeptides COR160, COR47, and COR15, respectively; a fourth cDNA, pHH29, has recently been shown to also encode a boiling-stable polypepetide (6.6 kDa) (Gilmour and Lin , unpublished). Since all *cor* cDNA clones derived from the differential screenings encode boiling-stable polypeptides, it appears that the transcripts encoding boiling -stable polypeptides are the most abundant *cor* gene products in *Arabidopsis*.

The accumulation of transcripts encoding boiling-stable polypeptides is not unique to *Arabidopsis*; it also occurs in cold-treated spinach and three *Solanum* species. It is possible that the induction of *cor* genes encoding boiling-stable polypeptides will be proven to be a common low temperature response in plants. Indeed, studies have

indicated that this response also occurs in winter wheat, and one of the wheat *cor* genes, encoding a boiling-stable polypeptide of 39 kDa, was found to be a homolog of *Arabidopsis cor47* (Lin *et al.*, 1990; Guo and Thomashow, 1992). Given the evolutionary distance between *Arabidopsis* and wheat, it would seem likely that related *cor* genes encoding boiling-stable polypeptide will be found in additional plant species. Future studies will determine whether this is true and whether any of the other *Arabidopsis cor* genes have counterparts in other plant species.

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. Otherwise, it would be hard to explain why 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 the accumulation of *cor* transcripts encoding the boiling-stable polypeptides would occur in a variety of plant species including wheat, and one of the wheat *cor* genes is actually homolog to the *Arabidopsis cor47* gene (Lin *et al.*, 1990; Guo and Thomashow, 1992). The more likely situation might be that the *cor* genes encoding boiling-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 (Volger and Heber, 1975). These investigators have reported that cold acclimated spinach synthesizes polypeptides that, on a molar basis, are greater than 10,000 times more effective in protecting thylakoid membranes against freezing damage (*in vitro*) than are known low molecular weight cryoprotectants such as sucrose and glycerol. Intriguingly, these polypeptides appear to have distinctive features in common with the *Arabidopsis* 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 of the *Arabidopsis cor* cDNA clones indicates that *Arabidopsis* COR polypeptides COR160, COR47, COR15, and COR6.6 are all hydrophilic (Lin and Thomashow, 1992; Gilmour *et al.*, 1992; Gilmour, McLarney, and Horvath, unpublished). Given these similarities, the possibility is raised that the *Arabidopsis* COR polypeptides are analogs of the spinach cryoprotective polypeptides. Whether the *Arabidopsis* COR polypeptides have cryoprotective properties and whether they contribute significantly to the freezing tolerance of cold-acclimated plants remain to be tested.

A second observation presented in this chapter is that the accumulation of transcripts encoding boiling-stable polypeptides is also a response of plants to drought stress. The drought-stressed *Arabidopsis* plants accumulated transcripts encoding all four boiling-stable COR polypeptides, in addition to a number of boiling-stable polypeptides that were not found in cold-treated plants. This observation leads to intriguing speculation that plants may have common genetic mechanisms for drought and freezing tolerance. 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 (Levitt, 1980; Sakai and Larcher, 1987; Steponkus and Lynch, 1989). Freezing tolerance must, therefore, include tolerance to dehydration. In support to this notion is the fact that drought stress has been observed to increase the freezing tolerance of wheat, rye (Siminovich and Cloutier, 1983), and cabbage (Cox and Levitt, 1976). Given this, it is reasonable to hypothesize that drought and freezing tolerance might involve related genetic mechanisms and gene products. In this regard, it is of interest to compare COR polypepetides with another family of heat-stable

polypeptides in plants, the LEA (late-embryogenesis-abundant) proteins (Dure *et al.*, 1989). LEA proteins are normally produced late in embryogenesis just prior to seed desiccation (Dure *et al.*, 1983) and in certain cases, they have been shown to be synthesized in plant seedlings in response to water stress (Close *et al.*, 1989; Mundy and Chua, 1988). It has been suggested that the LEA proteins might have a role in desiccation and drought tolerance (Baker *et al.*, 1988; Close *et al.*, 1989). Like the boiling-stable COR polypeptides, LEA proteins remain soluble upon boiling (Close *et al.*, 1989; Jocobsen and Shaw, 1989;) and are very hydrophilic (Close *et al.*, 1989; Dure *et al.*, 1989; Jacobsen and Shaw, 1989; Mundy and Chua, 1988). The structure similarities of COR polypeptides and LEA proteins are further demonstrated by the fact that *Arabidopsis* COR47 (Gilmour *et al.*, 1992) and its wheat counterpart (Guo and Thomashow, 1992) both had the lysine rich repeat that is characteristic to the Group II LEA proteins (Dure *et al.*, 1989). 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 needed to test the validity of this hypothesis.

A final point regarding the COR polypeptides described above is that all the data presented here only demonstrate the accumulation of *cor* transcripts in cold -acclimated plants. To be functionally relevant, the accumulation of COR polypeptides needs to be studied in cold-acclimated plants. It is known that COR160, COR47, as well as COR24 accumulate in such plants (Gilmour, *et al.*, 1988; Gilmour and Hajela, unpublished). It is not known, however, if the accumulation of the COR15 polypeptide occurs in the plants. Neither is the subcellular compartmentalization known for any of the COR polypeptides identified in *Arabidopsis* and other plants (Thomashow, 1990). These basic questions will be addressed in the following chapters.

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Chapter 3

ARABIDOPSIS GENE *cor15* ENCODES A POLYPEPTIDE HAVING POTENT CRYOPROTECTIVE ACTIVITY

SUMMARY

The cold-regulated gene of *Arabidopsis thaliana*, *cor15*, encodes a 15 kDa polypeptide designated COR15. In this chapter, I show that COR15 has potent cryoprotective activity in a standard *in vitro* cryoprotection assay. Specifically, COR15 was very effective in protecting the freezing-labile enzyme lactate dehydrogenase against freeze-inactivation; on a concentration basis, it was about 10^6-times more effective than sucrose and 10^2-10^3 times more effective than other proteins including bovine serum albumin. The possible role of *cor15* in cold acclimation is discussed.

INTRODUCTION

In many species of higher plants, a period of exposure to low nonfreezing temperature results in an increased level of freezing tolerance (Levitt, 1980; Thomashow, 1990). Considerable effort has been directed at understanding the molecular basis of this cold acclimation response, yet the mechanism remains poorly

understood. In 1970, Weiser (Weiser, 1970) suggested that cold acclimation might involve changes in gene expression. Since then, it has been clearly established that changes in gene expression occur during cold acclimation and cold-regulated genes have been isolated from a number of plant species (Thomashow, 1990). The roles that these genes have in the cold acclimation process, however, remain to be determined.

Heber and colleagues (Volger and Heber, 1975; Hincha *et al.*, 1989; Hincha *et al.*, 1990) have found that polypeptide fractions prepared from the leaves of cold -acclimated spinach and cabbage can prevent freeze-induced rupture of isolated thylakoid membranes. They have suggested that these polypeptides might have an important role in the enhancement of freezing tolerance that occurs with cold acclimation. The spinach and cabbage polypeptides, which appear to be cold-regulated (they could only be isolated from cold-acclimated plants), have molecular masses in the range of 10-30 kDa, are hydrophilic and remain soluble upon treatment with high heat. None of them, however, have been isolated to purity nor have the genes which encode them been isolated.

Three *Arabidopsis* cDNA clones representing *cor* (*co*ld-*r*egulated) genes have been identified to encode COR polypeptides that remains soluble upon boiling in aqueous solution (see Chapter 2). DNA sequence analysis of these cDNA clones indicated that the polypeptides they encode are hydrophilic (Lin and Thomashow, 1992; and Gilmour *et al.*, 1992). Thus, each of these *cor* genes encodes a polypeptide that has at least three characteristics in common with the cold-regulated polypeptides described by Heber and colleagues (Volger and Heber, 1975; Hincha *et al.*, 1989; Hincha *et al.*, 1990): cold-regulated expression, hydrophilicity, and "heat-stability." In addition, it has been found that one of these polypeptides, COR47, is related to certain LEA (late embryogenesis abundant) proteins (Gilmour *et al.*, 1992), a group of hydrophilic polypeptides (also boiling-stable) that have been hypothesized to have roles

in water-stress tolerance (Baker et al., 1988; Close et al., 1989; Jacobsen and Shaw, 1989). 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 (Levitt, 1980; Steponkus and Lynch, 1989). These and other considerations have led to the speculation that the boiling-stable *Arabidopsis* COR polypeptides might be analogues of the polypeptides described by Heber and colleagues (Volger and Heber, 1975; Hincha *et al.*, 1989; Hincha *et al.*, 1990) and that they might act as cryoprotectants by helping plant cells withstand the dehydration stress associated with freezing (Lin *et al.*, 1990). Here I show that COR15, the cold -regulated polypeptide encoded by *Arabidopsis* gene *cor15*, does indeed have potent cryoprotective activity in a standard *in vitro* assay.

MATERIALS AND METHODS

Preparation of COR15

cDNA clone pLCT10A and pLCT10B are described in the previous chapter. Since repeated attempts to express COR15 in *E. coli* with a number of expression vectors were unsuccessful (unpublished results), the COR15 polypeptide was prepared by *in vitro* transcription/ translation. pLCT10B was linearized by digestion with *Hin*dIII and the insert was transcribed *in vitro* with T7 RNA polymerase (Stratagene) using the T7 promoter carried on the pBluescript vector. The resulting transcripts were extracted with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with ethanol and in certain cases "capped" *in vitro* using the mCAP^{TM} capping kit (Stratagene). The resulting transcript was then translated *in vitro* using a rabbit reticulocyte lysate system with [^{35}S]methionine (Promega, Madison, WI). All these experiments were

carried out according to the manufacturer's instructions. The resulting translation mix containing [^{35}S]COR15 was boiled for 10 min and centrifuged (15 min in an Eppendorf centrifuge) to remove most of the proteins present in the *in vitro* translation mix. The boiling-soluble fraction, which contained the [^{35}S]COR15 polypeptide, was fractionated by SDS-PAGE (Laemmli, 1970) using 15% (w/v) polyacrylamide gels, and the COR15 polypeptide was located using a Betagen 603 Blot Analyzer (Betagen Corp.). The region of the gel containing the COR15 polypeptide was excised with a razor blade and the polypeptide recovered by electroelution as suggested by the manufacturer (CBS Science Inc., Del Mar, CA). The sample was then electrodialyzed against 20 mM Tris-HCl (pH 8.9) for 24 hours at 150 V, and precipitated with 3x volumes of acetone. The amount of COR15 obtained was estimated from the total dpm of the preparation, the specific activity of the [^{35}S]methionine used in the *in vitro* translation reaction, and the number of methionine residues per polypeptide as determined by DNA sequencing (see Chapter 4). The yields of COR15 in three experiments ranged from 0.4 μg to 1.5 μg.

As a control for the cryoprotection assays, mock preparations of COR15 were made using transcripts synthesized from the control plasmid pLCT10A. In this case, the region of the SDS-PAGE gel that corresponded to the position of the [^{35}S]COR15 produced in the test reactions was excised and treated the same way as the bona-fide COR15 samples.

Cryoprotection Assay

The cryoprotective activities of COR15, sucrose and various proteins were assayed essentially as described by Tamiya *et al.* (Tamiya et al., 1985). A solution of the freeze-labile enzyme L-lactate dehydrogenase (LDH) (EC 1.1.1.27, rabbit muscle lactate dehydrogenase-5[M4] isoenzyme type V-S; obtained from Sigma [St. Louis,

Mo]) was prepared (2.5 μg/ml) in 10 mM potassium phosphate buffer pH7.5. 50 μl of this solution was placed in a plastic microcentrifuge tube and 50 μl of the test compound suspended in 10 mM potassium phosphate buffer pH7.5 was added. The LDH/cryoprotectant mixtures were frozen at -20^{o}C for 24 h, thawed at room temperature for 5-10 min and assayed for enzyme activity as described by Tamiya *et al.* (Tamiya et al., 1985). Briefly, 20 μl of sample was added to 1 ml of assay mix (at room temperature) which contained 80 mM Tris-HCl pH7.5, 100 mM KCl, 2mM pyruvic acid and 0.3 mM NADH. The absorbance decrease at 340 nm was measured (at room temperature) using a RESPONSE recording scanning spectrophotometer (Gilford, Oberlin, Ohio). The rate of decrease in absorbance during the first 3 min of the reaction was used to calculate activity (rates were linear over this time interval). All samples were assayed in triplicate. The CP_{50} (50% cryoprotection) value was defined as the concentration of the additive required to give 50% residual LDH activity after the freeze-thaw cycle, and the average value of the means were compared. The proteins ß-galactosidase (ß-Gal; from *E. coli*), ovalbumin (from chicken egg), bovine serum albumin (BSA; Cohn fraction V, from bovine), and RNase A (type I-AS, from bovine pancreas) were purchased from Sigma.

RESULTS

The cryoprotective activity of COR15 was examined using the LDH freezing -inactivation assay, a well characterized model system commonly used in the studies of cryoprotectants (Greiff and Kelly, 1966; Soliman and Van Den Berg, 1970; Tamiya, et al., 1985; Carpenter and Crowe, 1988). COR15 and mock preparations of it were synthesized by *in vitro* translation of the *in vitro* transcription products of LCT10B and pLCT10A, respectively (Figure 3.1), and were purified by SDS-PAGE (see Materials

and Methods). The cryoprotective activity of the samples was then assessed by determining whether they could protect LDH against freeze-inactivation. Without addition of a cryoprotectant, a freeze/thaw cycle resulted in the LDH solution losing more than 90% of its enzymatic activity (not shown). Addition of the mock preparations of COR15 had no detectable effect; LDH still lost more than 90% of its enzymatic activity after a freeze/thaw cycle (not shown). However, addition of COR15 at a concentration of approximately 1.0 μg/ml resulted in almost complete protection of LDH against freeze-inactivation (Figure 3.2).

The results of the LDH cryoprotective assay are shown in Figure 3.2 and summarized in Table 3.1. Figure 3.2 represents a single experiment that shows the cryoprotective effects of different agents at varying concentrations, while Table 3.1 summarizes the CP_{50} values of the different agents from the number of experiments indicated. A comparison of the protective effect of COR15 with that of other agents indicated that COR15 has relatively potent cryoprotective activity. Whereas sucrose, a compound that is commonly regarded as an effective cryoprotectant and protein stabilizer (Lee and Timasheff, 1981; Carpenter and Crowe, 1988), had a CP_{50} of greater than 100 mg/ml ($8x10^8$ nM), the CP_{50} of COR15 was about 0.1 μg/ml (5.6 nM) (Figure. 3.2, Table 3.1). Thus, on a concentration basis, COR15 was approximately 10^6 times more effective than sucrose in protecting lactate dehydrogenase from freeze-inactivation. COR15 was also more effective in protecting LDH against freeze-inactivation than were a number of other proteins. For example, BSA, a protein which has been shown to be effective in protecting LDH against freeze -inactivation (Tamiya et al., 1985; Greiff and Kelly, 1966), had a CP_{50} of 28 μg/ml

Table 3.1: CP_{50} values of various agents[a]

Agents	MW	n	CP_{50} (μg/ml)	CP_{50} (nM)
Sucrose	340	4	270,000	800,000,000
Ovalbumin	45,000	4	84	1,900
ß-Gal.	116,000	4	160	1,400
RNase A	13,700	3	16	1,200
BSA	66,000	1	28	400
COR15	15,000	4	0.083	5.6

[a] The CP_{50} values were determined as described in the Materials and Methods. Standard errors (the standard deviation of a mean which was calculated as (s.d.)/($n^{1/2}$) for the CP_{50} shown are: Sucrose ± 9.3%; Ovalbumin ± 15%; ß-Gal ± 13%; RNase A ± 4.5% and COR15 ± 6.0%. n = the number of the experiments performed.

Figure 3.1 *In vitro* synthesis of COR15. The boiling-stable fractions of the *in vitro* transcription/translation products of pLCT10B (lanes 1-4) and pLCT10A (lanes 5-8) were fractionated on a 15% SDS-polyacrylamide gel, the gel was sealed in a plastic bag, and scanned for 50 min using a Betagen blot analyzer. The RNAs used in the *in vitro* translation were 0.05 μg (lanes 1 and 5), 0.1 μg (lanes 2 and 6), and 1 μg (lanes 3, 4 and 7, 8), and they were either translated directely (lanes 1-3 and 4-7) or capped *in vitro* before translation (lanes 4 and 8).

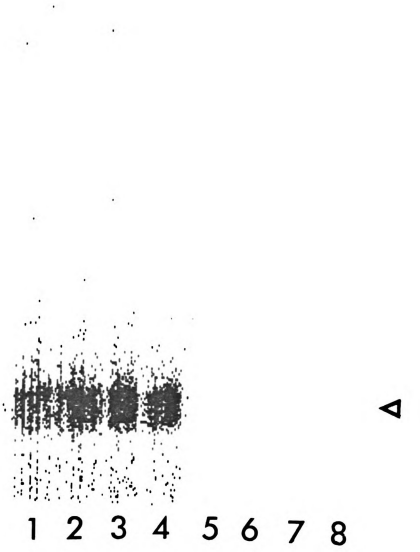

Figure 3.1

Figure 3.2 Cryoprotection of LDH. The curve shows the percentage LDH activity remaining after a freeze/thaw cycle in the presence of different concentrations of COR15 (open circles), BSA (solid circles), ovalbumin (solid squares), and sucrose (open squares).

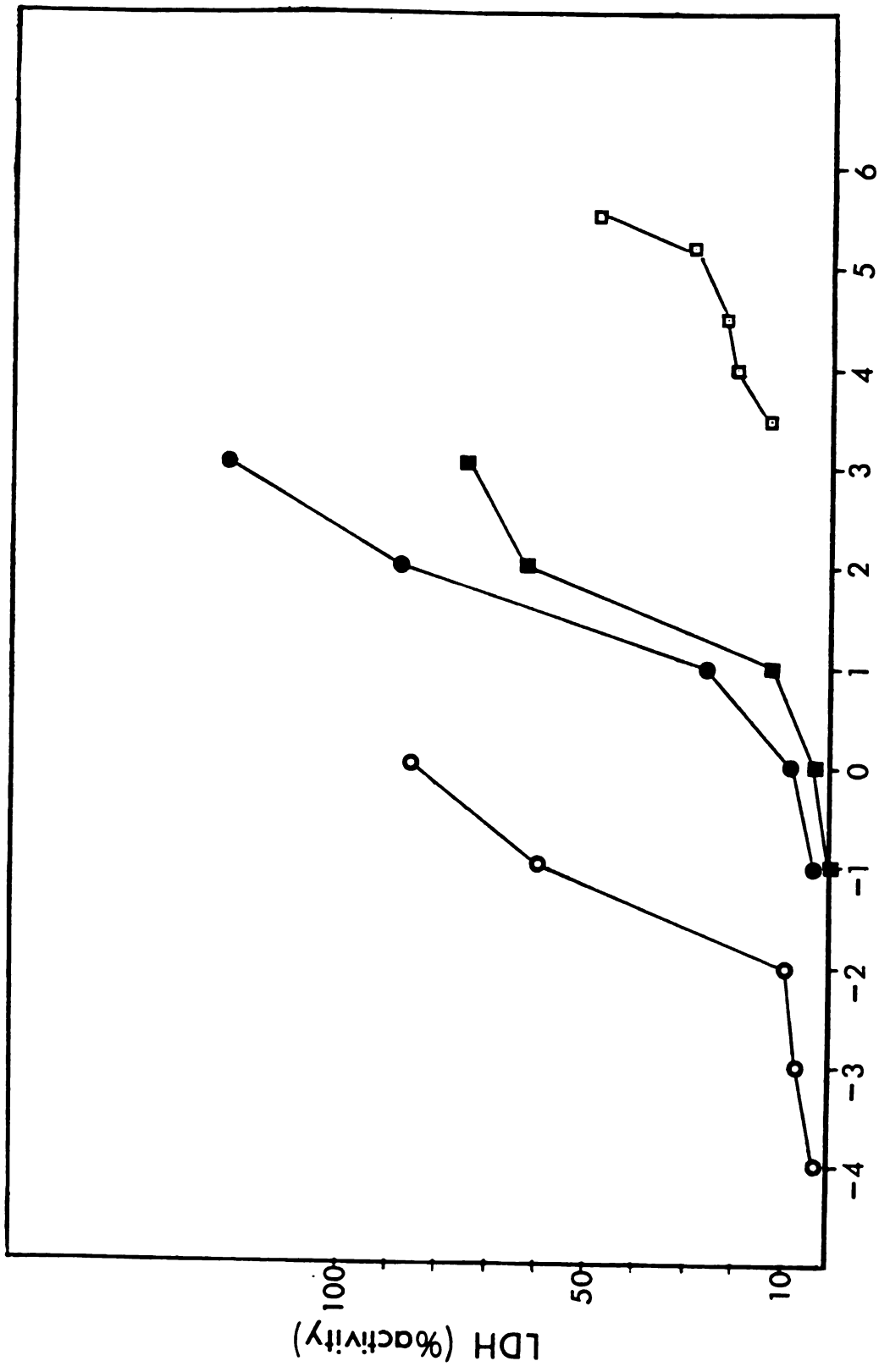

Figure 3.2

($4x10^2$ nM) (Figure 3.2, Table 3.1), a value that was about 100-fold higher than that of COR15. Other proteins, picked either randomly or because they were known to be hydrophilic like COR15 (i.e., RNase A) gave CP_{50} values ranging from approximately 300 to 2000-fold higher than COR15 (Table 3.1). Thus, on a concentration basis, COR15 was some 10^2 to 10^3 times more effective than BSA and other proteins in protecting LDH against freeze-inactivation.

DISCUSSION

It has been hypothesized that at least some of the boiling-stable polypeptides encoded by *Arabidopsis cor* genes might act as cryoprotectants in cold acclimated plants (Thomashow, 1990). The results presented here are consistent with this notion. Specifically, the data indicate that COR15, the boiling-stable polypeptide encoded by *Arabidopsis cor15*, has potent cryoprotective activity in a standard *in vitro* cryoprotection assay. The fact that COR15 demonstrated activity in the assay is not unexpected as it is generally observed that increased protein concentration stabilizes proteins against denaturation by many factors (Greiff and Kelly, 1966; Shikama and Yamazaki, 1966). However, what is significant is that COR15 appears to have particularly effective cryoprotective activity; on a concentration basis, it was about 10^2 times better in protecting LDH against freeze-inactivation than was BSA, a protein that is recognized as being an effective cryoprotectant and protein stabilizer (Tamiya et al., 1985; Greiff and Kelly, 1966).

While the data presented here encourage the notion that certain of the boiling -stable polypeptides encoded by *Arabidopsis cor* genes act as cryoprotectants, the hypothesis is, of course, far from proven. First of all, the relevance of the

cryoprotective feature of COR15 *in vitro* to its function *in vivo* is uncertain. Indeed, there is no direct evidence that these polypeptides contribute significantly to freezing tolerance in cold-acclimated plants. In addition, as described in the following chapter, the COR15 polypeptide is processed and targeted to the chloroplasts. Whether the mature polypeptide, COR15m, has greater, lesser or the same activity as COR15 in the LDH cryoprotection assay remains to be determined. More importantly, it remains to be established whether COR15m has a role in protecting chloroplasts against freeze -induced damage.

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Chapter 4

CHARACTERIZATION OF THE *ARABIDOPSIS cor15* GENE PRODUCT

SUMMARY

In the previous chapter I showed that *cor15* encodes a boiling-stable polypeptide, COR15, which has potent cryoprotective activity in an *in vitro* assay. In this chapter, cDNAs for *cor15* were characterized by DNA sequence analysis. The data indicated that *cor15* encodes a 14.6 kDa hydrophilic polypeptide. The N-terminal amino acid sequence of this polypeptide closely resembles transit peptides that target proteins to the stromal compartment of chloroplasts. Immunoblot analysis indicated that COR15 is processed *in planta* and that the mature polypeptide, COR15m, is present in the soluble fraction of chloroplasts. The biochemical activity of COR15m is not known, however, a strong intermolecular interaction of COR15m with itself is suggested by the observation that COR15m can form multimers even in the presence of SDS.

INTRODUCTION

It is now clear that changes in gene expression occur during cold acclimation in a wide range of plant species (Weiser, 1970; Guy, 1990; Thomashow, 1990). It is not yet 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. While no functions have yet been determined for these genes, results have been intriguing. Mohapatra *et al.* (Mohapatra *et al.*, 1989), for example, have found that the levels of expression of three cold-regulated genes from alfalfa correlate positively with the freezing tolerances of four different alfalfa cultivars. Further characterization of these cDNA clones, however, has not been reported. Another approach to study the function of the cold-regulated genes in plant cold acclimation is to analyze the amino acid sequence of COR polypeptides. For example, one of the *Arabidopsis* cold-regulated genes, *kin1*, has been reported to encode an alanine-rich polypeptide that has amino acid sequence similarities with certain fish antifreeze or thermal hysteresis proteins (Davies and Hew, 1990). Whether KIN1 has antifreeze properties *in vitro* or *in planta* is not known.

In the previous chapters, it has been established that in *Arabidopsis* and some other plants, cold acclimation results in the accumulation of transcripts encoding polypeptides that share the unusual property of remaining soluble upon boiling in aqueous solution. *Arabidopsis* genes encoding the boiling-stable COR160, COR47, and COR15 polypeptides have been identified to be represented by the cDNA clones pHH28, pHH7.2, and pLCT10, respectively. Moreover, one of the COR polypeptides, COR15, has been found to have potent cryoprotective activity in an *in vitro* assay. In this chapter, COR15 is further characterized by DNA sequence analysis of cDNAs for

cor15. The accumulation of the *cor15* gene product in cold-acclimated *Arabidopsis* is also investigated by immunological analysis.

MATERIALS AND METHODS

Plant Material

Arabidopsis thaliana ecotype RLD was grown in clay pots in planting soil (Baccto Co., TX) with a photoperiod of 14 h or 18 h. The photoperiod was 14 h if the vegatative tissue was needed, and it was 18 h if the plants were used for seed production. For cold acclimation, 2 to 3 week old plants were transferred to a cold room with a temperature of 4^{o}C at soil level for 3 days. Other conditions were the same as described in Chapter 2.

DNA Sequencing

DNA sequences of the cDNA inserts of clones pHH71.1, pLCT10A and part of pHH67 were determined on either single or double stranded DNA templates by the dideoxy chain termination method (Sanger *et al.*, 1977) using Sequenase^{TM} (U. S. Biochemical) according to the manufacturer's instructions. Single stranded plasmid DNA was prepared from *E. coli* strand MV1190 using the helper phage M13K07 (Vieira and Messing, 1987). Double stranded sequencing was performed according to the method of Zhang *et al* (Zhang *et al.*, 1988). Deletions of the cDNA insert in pBluescript SK(-) were generated by digestion with exonuclease III and mung bean nuclease (Henikoff, 1987). The complete sequence of each strand of the inserts in pHH71.1 and pLCT10A was determined. Nucleotide and amino acid sequence analysis was performed using the DNAsis and PROsis programs of Hitachi (Hitachi Engineering

Co., Ltd) and the CGC (University of Wisconsin Genetics Computer Group) programs (version 6.0) of the University of Wisconsin Biotechnology Center (Madison, WI).

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 *Eco*RI cDNA insert of pLCT10B into the *Eco*RI site of pRIT2T, a protein A fusion vector (Nilsson *et al.*, 1985) (Pharmacia). The recombinant plasmid was transformed into *E. 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 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 IgG 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 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^{TM} 10 filter (Amicon). Analogous procedures utilizing the unmodified pRIT2T vector were employed to obtain the protein A polypeptide (26 kDa). New Zealand white rabbits were immunized with the protein preparations by subcutaneous injection of 80 μg protein and boosted once after 4 weeks with the same amount of protein.

Antiserum recognizing COR160, an *Arabidopsis* cold-regulated boiling-stable polypeptide, was obtained from Sarah Gilmour (S Gilmour, M Thomashow, unpublished). Antisera to carbonic anhydrase (Fawcett *et al.*, 1990) and glycolate

oxidase (Volokita and Somerville, 1987) of spinach were kindly provided by Chris Somerville.

Chloroplast Isolation

Chloroplasts were isolated by modification (S Hugly, C Somerville, unpublished) of a previously described procedure (Somerville *et al.*, 1981). Leaves (10 g) harvested from 2-3 week old plants (rosette stage) were immersed in ice water for 5 min, blotted dry, placed in 150 ml 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 sec in a Tissumizer (Tekmar, Cincinnati, OH) at maximum speed. The homogenates were passed through Miracloth (Calbiochem) and centrifuged at 1400g for 1 min. Pelleted material was gently resuspended with a camel hair paint brush in 2 ml resuspension buffer [100mM Tris (pH7.9), 300 mM glycerol, 1 mM $MgCl_2$, 1 mM DTT], layered onto a discontinuous percoll gradient [1 ml 60% (v/v) percoll, 10 ml 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 5x volume of resuspension buffer and collected by centrifugation at 1300g for 1 min.

Preparation of Protein from *Arabidopsis*

Total soluble protein was prepared by grinding plant tissue in liquid N_2 followed by grinding in extraction buffer [50 mM Tris (pH 8.0), 5 mM EDTA, 2.5% (w/v) polyvinylpolypyrrolidone] (4 ml buffer/gram tissue). The material was then centrifuged (10,000g, 10 min), the pellet discarded, and the proteins in the supernatant

collected either by ammonium sulfate precipitation or by addition of 3 vol acetone followed by centrifugation (14000g for 10 min).

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

Gel Electrophoresis of Proteins

There were three types of the polyacrylamide gel electrophoresis used for the experiments described in this chapter: SDS-PAGE, native PAGE, and tricine-SDS -PAGE. SDS-PAGE was as described (Laemmli, 1972). Native PAGE was prepared and performed basically the same as with Laemmli gels except that no SDS was included in any solutions. Tricine-SDS-PAGE was essentially according to the method of Schagger and Von Jagow (Schagger and Von Jagow, 1987), where tricine was used as the trailing ion in replace of the glycine that is used in the Laemmli gel, and breifly described as follows. The separation gel (10% acrylamide, 0.6% bisacrylamide, 1 M Tris-HCl pH 8.5, 0.1% SDS) was overlaided with a layer of stacking gel (3.84% acrylamide, 0.23% bisacrylamide, 1 M Trise-HCl pH 8.5, 0.1 % SDS); protein samples were disolved in the same SDS sample buffer for SDS-PAGE (Laemmli, 1972), and electrophoresis was conducted with the anode buffer (0.2 M Tris-HCl pH 8.9) and the cathode buffer (0.1 M tricine, 0.1 M Tris base, 0.1% SDS) at a constant current.

Immunoblot Analysis

Proteins were fractionated by SDS-PAGE and transferred to nitrocellulose (Schleicher and Schuell) as described (Towbin *et al.*, 1979). Immunoblots were then treated with antisera and bound antibody detected using protein A-conjugated alkaline phosphatase (Sigma) as described (Blake *et al.*, 1984).

Preparation of A Putative COR15m Recombinant Polypeptide

The putative COR15m is a polypeptide consisting of the amino acids 51 to 139 of COR15 (see Results). A pair of primers was synthesized in the Macromolecular Structural Facility in the Biochemistry Department of Michigan State University. Primer I, (5' *TCTCCATGGCTAAAGGTGACGGC* 3'), corresponds to nucleotides 209 -231 of the cDNA insert of pLCT10 with 4 mismatches (underlined). The mismatches in Primer I result in the generation of the restriction site *Nco*I and a methionine codon at the 5' end of the primer. Primer II (3' *ACGGTGTTTCATCCCTAGGTGG* 5'), corresponds to nucleotides 474-495 with 4 mismatches (underlined). The mismatches in Primer II result in the generation of the restriction site *BamH*I at the 5' end of the primer. The DNA fragment corresponding to nucleotides 209-495 of the pLCT10 insert was synthesized using the primers and standard PCR protocols (Innis *et al.*, 1990), then end-repared, and cloned into the *Sma*I site of pBluescript by standard recombinant DNA techniques (Sambrook *et al.*, 1989). This DNA fragment has an open reading frame encoding the putative COR15m plus a methionine at the N -terminus; this polypeptide is referred to as recombinant COR15m. The *Nco*I/*BamH*I fragment of the resulting plasmid (pLCT10m) was isolated and cloned into the expression vector pET9d (Novagen, Madison, WI) carrying the bacteriophage T_7 RNA polymerase promoter (Rosenberg *et al.*, 1987). The resulting plasmid (pLCT103) was transformed into *E. coli* strain BL21 (DE3, plysS), the expression of the protein

induced by IPTG, and cells harvested and lysed according to the supplier's instructions. The lysates were boiled, the insoluble material removed by centrifugation, and the recombinant COR15m polypeptide was purified using preparative tricine-SDS-PAGE. Antiserum was raised against COR15m as described for the protein A-COR15 fussion protein. The relative migration of COR15m and the recombinant COR15m were analyzed by tricine-SDS-PAGE.

RESULTS

DNA Sequence Analysis of cDNAs for COR15

The DNA sequences of three cDNA inserts representing COR15 were determined. The data for the cDNA insert in pLCT10A is shown in Figure 4.1. The data indicate that the insert has an open reading frame that could encode a 139 amino acid long polypeptide with a predicted molecular weight of 14,604 daltons, a value consistent with the *in vitro* transcription/ translation experiment presented in Chapter 2. The gene encoding this polypeptide has been designated *cor15*. The deduced polypeptide, designated COR15, had a high alanine (17.9 mol%), lysine (14.3 mol%), and glutamic acid (9.3 mol%) content and was devoid of cysteine, tryptophan and proline residues (Table 4.1). 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 (Figure 4.2). Analysis of the potential secondary structure of the polypeptide using the algorithm of Robson (Garnier *et al.*, 1978) indicated that the latter two thirds of the polypeptide (from residue 56 to 131) was likely to assume an α-helical configuration (Figure 4.3). 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

Figure 4.1. Nucleic acid sequence of the cDNA insert in pLCT10A and the deduced amino acid sequence of COR15. The loosely defined consensus cleavage site for chloroplast transit peptides (Gavel and Heijne, 1990) is double-underlined and the predicted site of cleavage is indicated by an asterisk (see text). The predicted amino acid sequence for COR15m following the alanine residue is underlined. The two nucleotides underlined indicates the location of the 306 bp intron in the insert of pHH71.1.

```
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
1                            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 Ile Tyr Ala*Ala 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 ATG TTT TGC AAA   672

673  ATA TTC AAT AGA CAA ATT AAA TAA AAA AAA AAA AAA          3'      708
```

Figure 4.1

Table 4.1 Amino acid composition of COR15 and COR15m

Amino Acid	Mol% (COR15)	Mol% (COR15m)
Glycine (G)	8.57	7.78
Alanine (A)	17.86	21.11
Valine (V)	7.14	4.44
Leucine (L)	4.29	4.44
Isoleucine (I)	1.43	1.11
Serine (S)	7.86	2.22
Threonine (T)	5.76	6.67
Cysteine (C)	0.00	0.00
Methionine (M)	2.14	0.00
Aspartic acid (D)	6.43	10.00
Asparagine (N)	2.86	4.44
Glutamic acid (E)	9.29	14.44
Glutamine (Q)	2.86	0.00
Arginine (R)	1.43	0.00
Lysine (K)	14.29	17.78
Histidine (H)	0.71	0.00
Phenylalanine (F)	4.29	2.22
Tyrosine (Y)	2.14	2.22
Tryptophan (W)	0.00	0.00
Proline (P)	0.00	0.00
Molecular weight	14604.51	9352.66
Isoelectric point	7.52	4.56

Figure 4.2. Hydropathy profile of COR15. Plots are according to Kyte and Doolittle (Kyte and Doolittle, 1982) using a window of 9 residues. Negative values indicate hydrophilic regions. Numbers on the abscissa indicate amino acid residues.

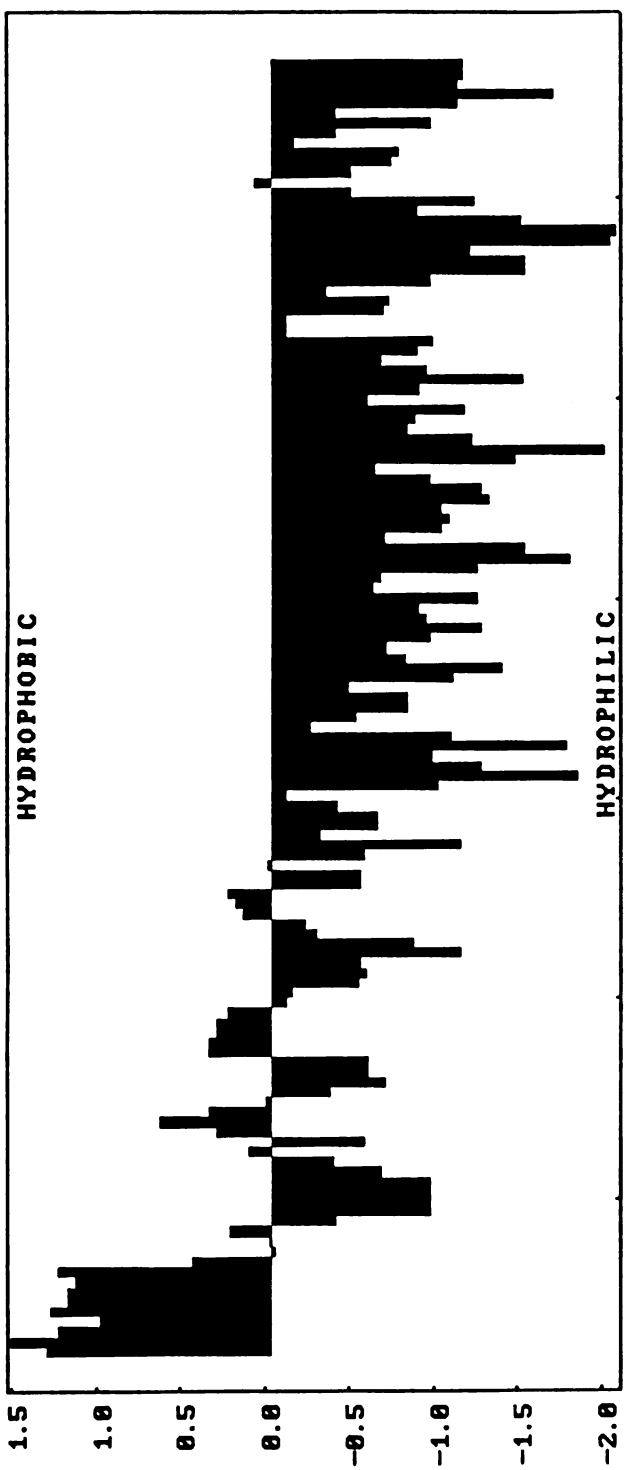
HYDROPHOBIC
HYDROPHILIC
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
20
40
60
80
100
120

Figure 4.2

Figure 4.3. The predicted secondary structure of COR15m according to Garnier *et al* (Garnier *et al.*, 1978).

```
          10        20        30        40        50        60        70
      MAMSFSGAVLTGMASSFHSGAKQSSFGAVRVGQKTQFVVVSQRKKSLIYAAKGDGNILDDLNEATKKASD

HELIX         H                                    HHHHHHH   HHHHHHHHHHHHHHH
SHEET          SS               SSSSS    SSSSSS
TURN               T       TT          TTT      TTTT        TT
COIL             CC CCCCCCC  CCC     C                    C

          80        90       100       110       120       130       140
      FVTDKTKEALADGEKAKDYVVEKNSETADTLGKEAEKAAAYVEEKGKEAANKAAEFAEGKAGEAKDATK*

HELIX HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH
SHEET
TURN
COIL
```

Figure 4.3

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.

A previously described cDNA clone, pHH67 (Hajela *et al.*, 1990), was found to be a homolog to pLCT10 by restriction mapping, southern blot analysis, and partial DNA sequence (not shown). The cDNA insert of pHH67 is 26 bp shorter at the 5' end than that of pLCT10. The DNA sequence of another cDNA clone, pHH71.1 was also determined and is shown in Figure 4.4. Analysis of the DNA sequence of pHH71.1 revealed that it had an insert of 306 bp within the coding sequence for COR15 (Figure 4.4). This same sequence is in a genomic clone for *cor15* and thus is an intron (Wilhelm, unpublished).

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 kDa from *in vitro* translations of RNA isolated from cold acclimated *Arabidopsis* (Figure 4.5A) and it precipitated the COR15 polypeptide prepared by *in vitro* transcription\translation of pLCT10B (Figure. 4.5B). Antiserum prepared against protein A alone did not precipitate the 15 kDa polypeptide in either sample (Figure 4.5A and 4.5B).

Processing of COR15 *In Planta*

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, fractionated by ammonium sulfate precipitation and subjected to SDS-PAGE. Immunoblots were then prepared and treated with the COR15 antiserum.

Figure 4.4. Nucleic acid sequence of the cDNA insert in pHH71.1. The underling indicates the putative 306 bp intron.

5′

```
1    CAAAAACTTC TTTTTATTCT CACATCTTAA AGATCTCTCT CATGGCGATG TCTTTCTCAG   60
61   GAGCTGTTCT CACTGGTATG GCTTCTTCTT TCCACAGCGG AGCCAAGCAG AGCAGCTTCG  120
121  GCGCTGTCAG AGTCGGCCAG AAAACTCAGT TCGTCGTCGT TTCTCAACGC AAGAAGTCGT  180
181  TGATCTACGC CGCTAAAGGT GACGGCAACA TCCTCGATGA CCTCAACGAG GCCACGTAAG  240
241  TCTACATTCT TTCTTCTTTT AGTATCTTGC CTCATAAGTA AGGATCTTAG CAGGCAATGT  300
301  TTATGGTATA CTATATTAGT ATAGATTTTA GTGGAAATAT GTTTGTTTTG AACTTATTTT  360
361  ATGATCATAT TTGACTATTA TCAAAGATAA AGATTCATAT ACCGTACATT ATATATCTCT  420
421  ATTTTTCTAG TTTACATGTA TAGCTCTAAG TTTATTTGAT GATTCTGTTG ACTACTTTTG  480
481  GATATGTGTT TTGAAACCTT TGATAAATAC TAAAATAAAT TTTAATTTGA AAATGATATA  540
541  GAAAGAAAGC TTCAGATTTC GTGACGGATA AAACAAAAGA GGCATTAGCA GATGGTGAGA  600
601  AAGCGAAAGA CTACGTTGTT GAAAAAAACA GTGAAACCGC AGATACATTG GGTAAAGAAG  660
661  CTGAGAAAGC TGCGGCGTAT GTGGAGGAGA AAGGAAAAGA AGCCGCAAAC AAGGCGGCAG  720
721  AGTTCGCGGA GGGTAAAGCA GGAGAGGCTA AGGATGCCAC AAAGTAGGGT CTTACCTAAT  780
781  CAGTTAATTT CAAGCACTTA AACTCGTAGA TATATTGATC CATATCCTCT CTCTTCATGT  840
841  TTAATAGTAC TTACAATAAG ATGAGTCTGT TGTAATTTCT ATTAATTTCA CATCACAACT  900
901  GAAATAAGAT ATGGTATCCA CAGTCACCGT CAAAAAAAAA AAAAAAAAAA              950
```

3′

Figure 4.4

Figure 4.5. Antibody prepared against the protein A-COR15 fusion polypeptide recognizes COR15. (A) Poly(A^+) RNA isolated from cold acclimated plants was translated *in vitro*, the polypeptide products immunoprecipitated with either the antiserum (1/50 dilution) raised against protein A (AntiA) or the antiserum (1/50 dilution) 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 RNA 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 gels.

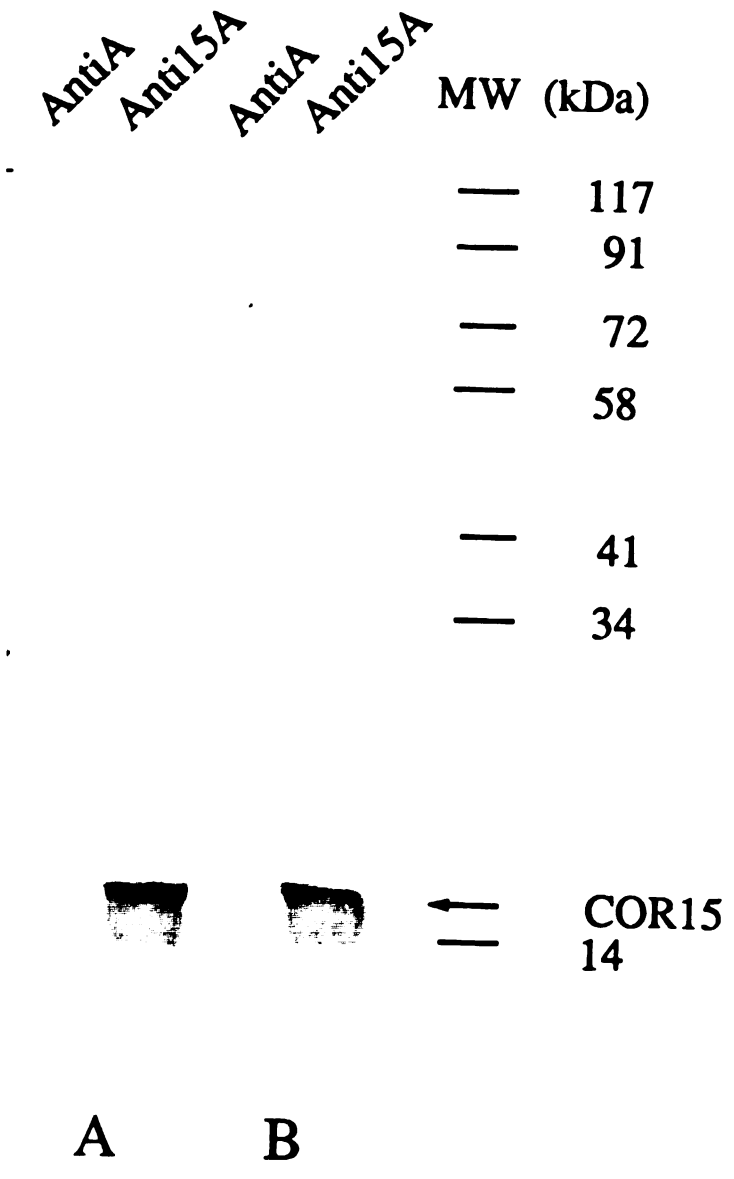
AntiA
Anti15A
AntiA
Anti15A
MW (kDa)
117
91
72
58
41
34
COR15
14
A
B

Figure 4.5

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 (Figure 4.6). The apparent molecular weight of the COR15 polypeptide, however, was only about 9 kDa, considerably smaller than the 15 kDa *in vitro* translation product (Figure 4.5). These data suggested that the COR15 polypeptide might be processed *in planta*; 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 (Gavel *et al.*, 1990). A comparison of the putative signal peptide of COR15 and common features of the transit peptide for chloroplast targeting is summarized in Figure 4.7. First, the sequence of COR15 at residues 48-51, Ile-Tyr-Ala-Ala, matches 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 weight of 9.4 kDa (Table 4.1), a size that is consistent with the COR15 product observed *in planta* (Figure 4.6). Second, chloroplast transit peptides typically have one or more arginine residues in position -6 to -10 relative to the putative cleavage site; COR15 had an arginine residue at position -8 (Figure 4.7). 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 has a serine plus threonine content of 22% and contains no

Figure 4.6. Evidence that the COR15 polypeptide is processed *in planta*. Total soluble protein prepared from 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 11-18% (w/v) gradient gel (32 cm long). The bottom half of the gel was cut to fit a conventional electroblot apparatus and the proteins transferred to nitrocellulose. The immunoblots were then treated with the protein A-COR15 antiserum (1/25 dilution) and bound antibody was detected as described in Materials and Methods.

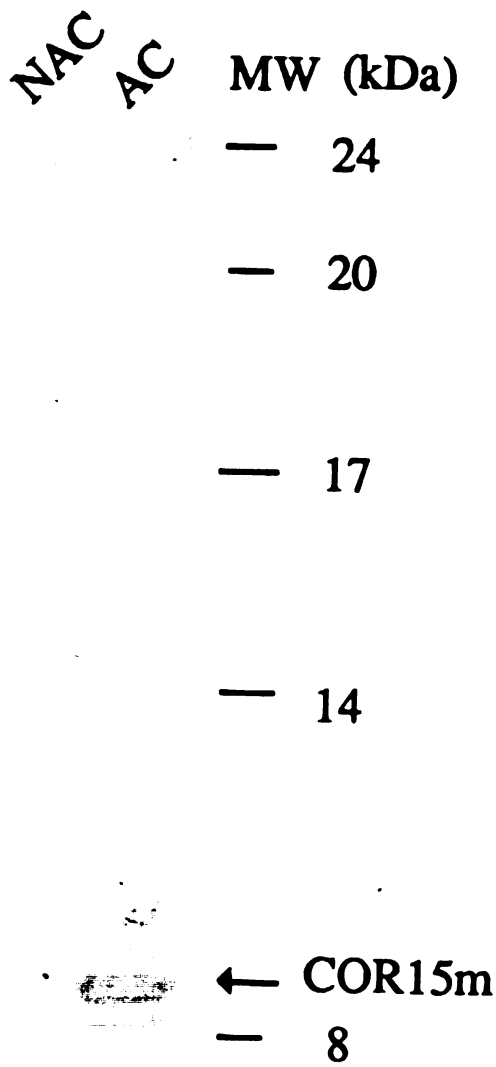

Figure 4.6

Figure 4.7. Comparison of the chloroplast targeting transit peptide (cTP) and the putative cTP of COR15.

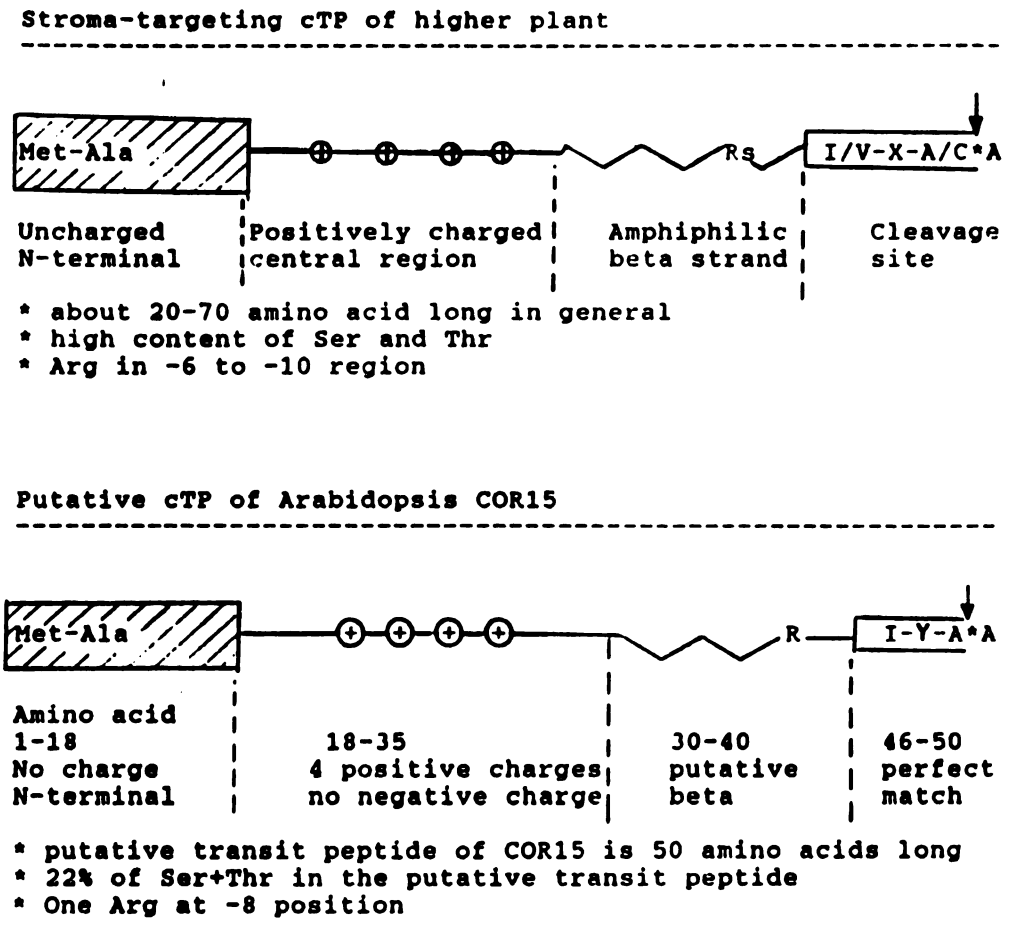
Stroma-targeting cTP of higher plant
Met-Ala
Rs
I/V-X-A/C*A
Uncharged N-terminal
Positively charged central region
Amphiphilic beta strand
Cleavage site
* about 20-70 amino acid long in general
* high content of Ser and Thr
* Arg in -6 to -10 region
Putative cTP of Arabidopsis COR15
Met-Ala
R
I-Y-A*A
Amino acid 1-18 No charge N-terminal
18-35 4 positive charges no negative charge
30-40 putative beta
46-50 perfect match
* putative transit peptide of COR15 is 50 amino acids long
* 22% of Ser+Thr in the putative transit peptide
* One Arg at -8 position

Figure 4.7

glutamic or aspartic acid residues (Figure 4.7). 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 (Garnier *et al.*, 1978). In agreement, COR15 residues 1-18 were without charge, residues 18-35 had 4 positive charges and no acidic residues, and residues 27-41 were predicted to form a beta-sheet (Figure 4.3 and Figure 4.7).

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 (Figure 4.8, lane 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 (Figure 4.8, lane 1). Chloroplasts were then purified from the leaves of cold acclimated plants, total soluble proteins prepared, and indeed, COR15m was detected in the extracts (Figure 4.8, lane 4). Similarly, carbonic anhydrase, a stromal chloroplast protein (Fawcett *et al.*, 1990), was detected in protein extracts prepared from whole leaves and chloroplasts (Figure 4.9A). In contrast, COR160 (Figure 4.9B), another cold-regulated boiling-stable polypeptide of *Arabidopsis* described previously, and glycolate oxidase (Figure 4.9C), a peroxisomal enzyme (Volokiao and Somerville, 1987), 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 (Figure 4.8, lane 2) or chloroplasts (Figure 4.8, lane 5) isolated from nonacclimated plants nor in membrane fractions prepared from chloroplasts isolated from either cold acclimated (Figure 4.8, lane 7) or nonacclimated plants (Figure 4.8, lane 6).

Figure 4.8. Detection of COR15m in leaves and chloroplasts isolated from cold acclimated plants. Various protein fractions were prepared from cold acclimated and nonacclimated plants and 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 (1/25 dilution) raised against the protein A-COR15 fusion and developed as described in Materials and Methods. The samples are: lane 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.

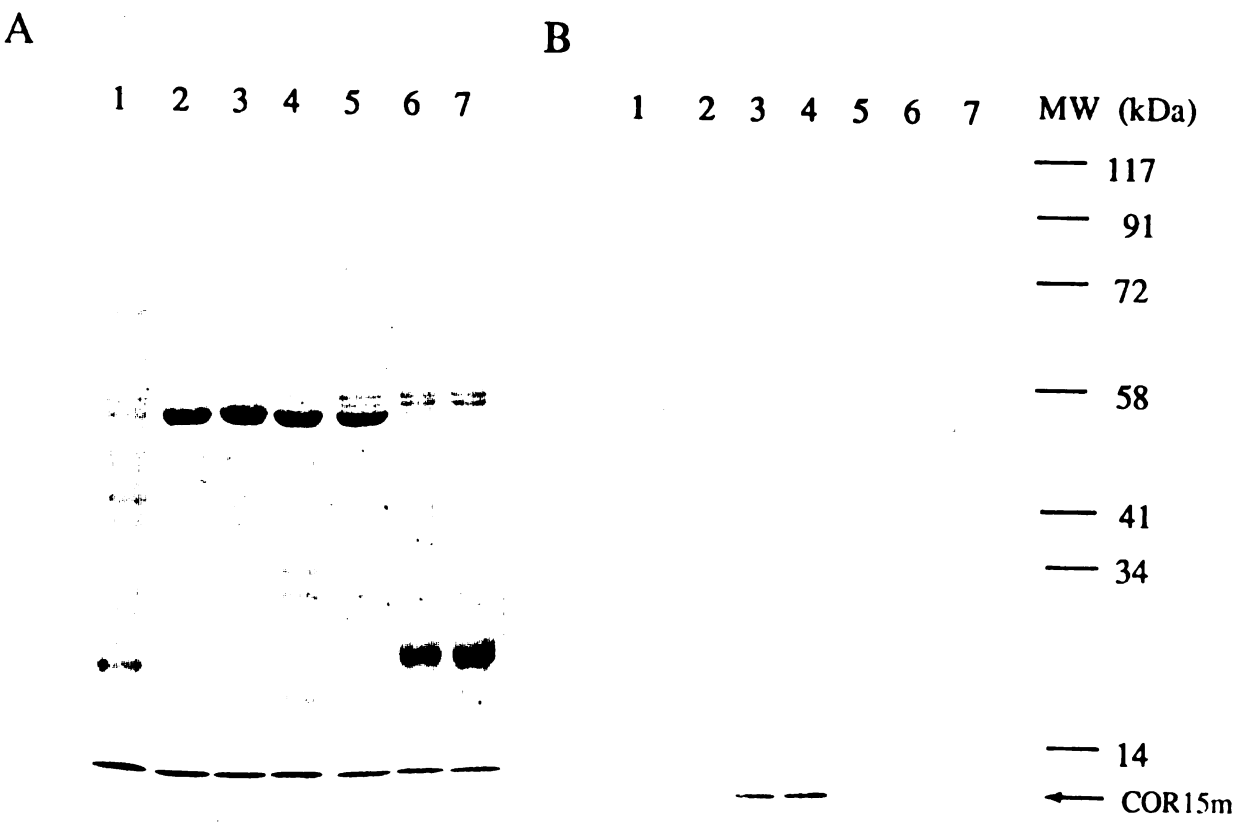

Figure 4.8

Figure 4.9. Detection of carbonic anhydrase, COR160, and glycolate oxidase in protein fractions isolated from nonacclimated and cold acclimated plants. Immunoblots were prepared from protein preparations fractionated by SDS-PAGE on 10% (w/v) gels. The transfers were then treated with antisera to either carbonic anhydrase (A), COR160 (B), or glycolate oxidase (C) and developed as described in Materials and Methods. The samples are: lanes 1 and 2, total protein prepared from chloroplasts isolated from nonacclimated (lane 1) and acclimated plants (lane 2), respectively; lanes 3 and 4, total soluble protein prepared from leaves of acclimated (lane 3) and nonacclimated (lane 4) plants, respectively.

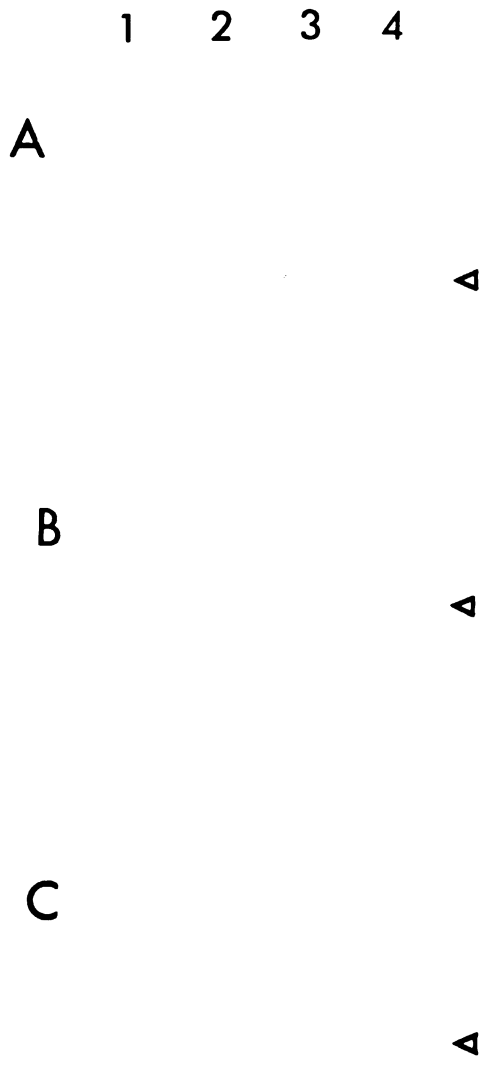

Figure 4.9

COR15m Comigrates on SDS-PAGE with Recombinant COR15m Expressed in *E. coli*

A gene that encodes the putative COR15m with the addition of a methionine residue at the N-terminal end was created using the polymerase chain reaction (see Materials and Methods). The gene was then cloned into the expression vector pET9d, expressed in *E. coli*, and the recombinant COR15m polypeptide purified (see Materials and Methods). The relative migration of the purified recombinant COR15m was compared with that of the authentic COR15m of *Arabidopsis* chloroplast on high resolution tricine-SDS-PAGE. This system has superior resolution to conventional glycine-SDS-PAGE (Laemmli, 1970) for proteins in the range of 5 to 25 kDa (Schagger and Von Jagow, 1987). An immunoblot analysis indicated that the recombinant COR15m migrated to the same position as the COR15m prepared from cold acclimated *Arabidopsis* (Figure 4.10). Thus, the data are consistant with COR15 being processed at the putative cleavage site of the chloroplast transit peptide (Figure 4.1 and Figure 4.7).

Intermolecular Interactions of Polypeptides COR15 and Recombinant COR15m

Preparations of COR15 polypeptide synthesized by *in vitro* transcription /translation did not migrate as a single band in the native PAGE (Figure 4.11A) as it did in the SDS-PAGE (Figure 4.11B). Instead, it migrated as multiple bands at much higher molecular weight positions, and the majority of the proteins barely migrated into the gel (Figure 4.11A). This could have resulted from COR15 binding to other unlabelled proteins in the translation system (rabbit reticulocyte lysate) or from it forming multimers itself. The abnormal migration of COR15 was not effected by the addition of nonionic detergents (Figure 4.11A). More interestingly, when the recombinant COR15m was purified as a single band from tricine-SDS-PAGE gels (see

Figure 4.10. Comigration of the chloroplast COR15m with recombinant COR15m. The gene encoding the putative COR15m with an additional methionine residue at the N-terminal was created, expressed in *E. coli*, and the recombinant COR15m purified as described in the Materials and Methods. (A). The boiling-stable proteins of the *E. coli* cell hosting vector plasmid pET9d (lane 4) or plasmid pLCT103 (lane 3), purified COR15m (lane 2) and the mock preparation (lane 1) were fractionated by tricin-SDS -PAGE on a 10% gel, and stained with Coomassie brilliant blue. (B). Soluble proteins of the chloroplast prepared from nonacclimated (lane 1) and 3-day-cold-acclimated (lane 2) *Arabidopsis*, and the recombinant COR15m (0.01 μg) (lane B.3) were fractionated by tricine-SDS-PAGE on a 10% gel, the bottom half of the gel blotted to nitrocellulose, and the immunoblot was developed with antiserum against COR15m (1:50 dilution). The arrow head indicates COR15m.

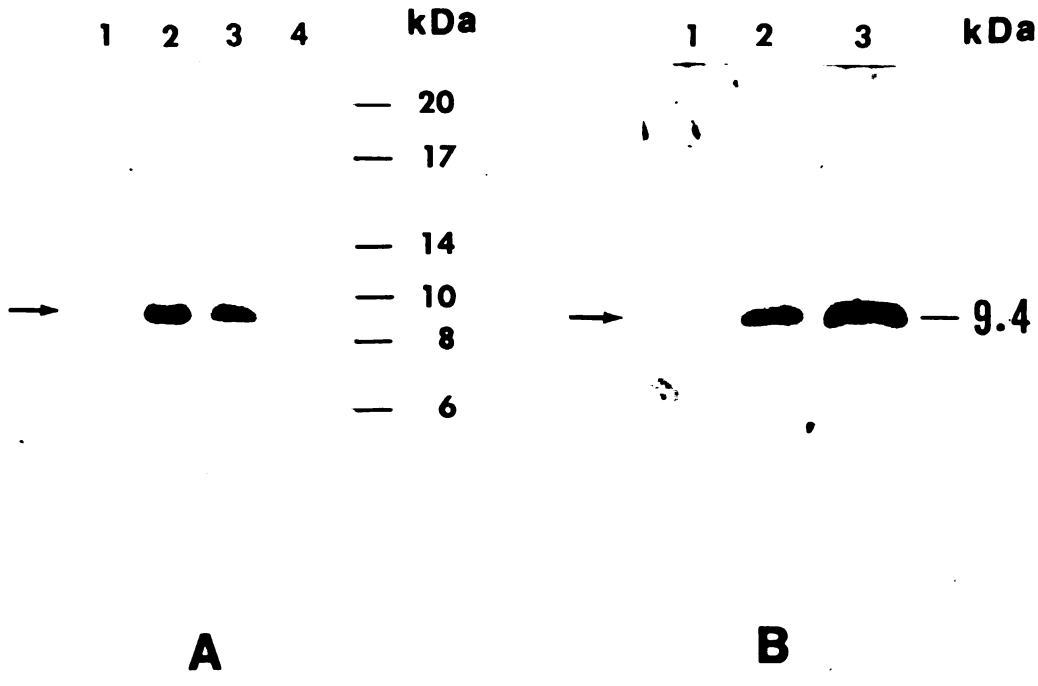

Figure 4.10

Figure 4.11. Migration of COR15 on a native polyacrylamide gel. [^{35}S]methionine -labelled COR15 was prepared by *in vitro* transcription/translation of pLCT10B as described in the Materials and Methods. Translation products were fractionated either by native PAGE on a 15% gel (A) or by SDS-PAGE on a 15% gel (B). Proteins were either fractionated directely (lanes A.1, A.6, B.1 and B.2), or incubated in the sample buffer containing nonionic detergent NP-40 of 0.1% (lane A.2) and 2% (lane A.3), or Triton X-100 of 0.1% (lane A.4) and 2% (lane A.5) at 65^{o}C for 15 min. Lane A.6 and B.2 show the transcription/translation of pLCT10A. The positions of the molecular weight markers are indicated for the panel A. The positions of 160, 47, 24, and 15 kDa COR polypeptides are indicated in panel B.

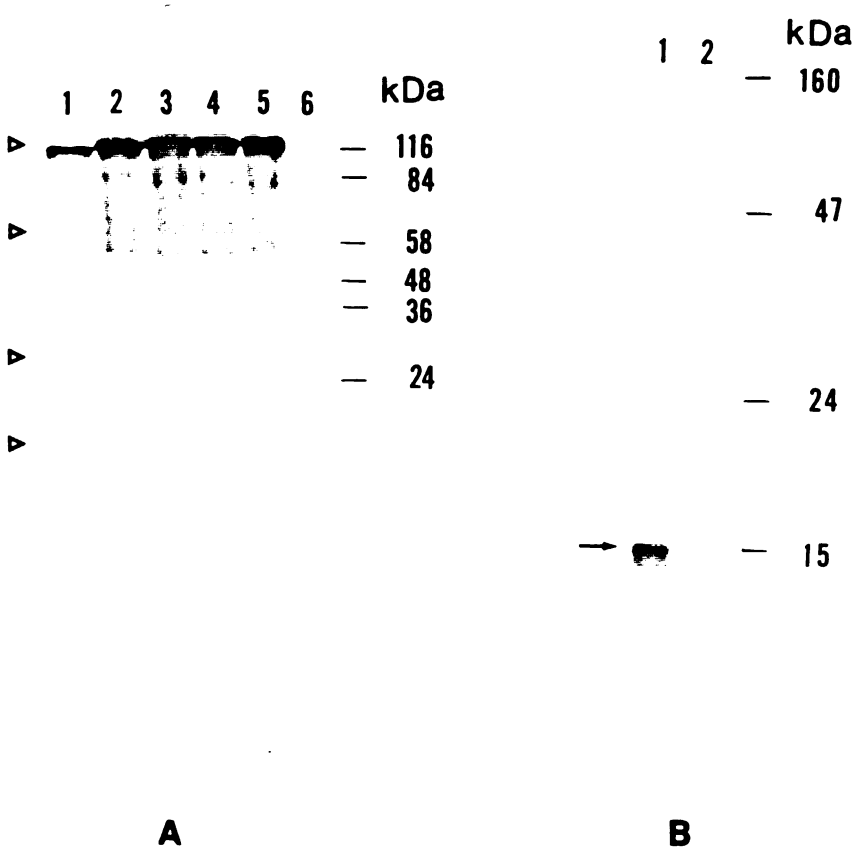

Figure 4.11

Figure 4.12. Immunoblot showing the formation of multimers of recombinant COR15m. The recombinant COR15m was purified as a single band from SDS-PAGE (see Materials and Methods), fractionated on a 11% tricine-SDS-PAGE, and the immunoblot prepared with antiserum against the recombinant COR15m (1:50 dilution) as described in the Materials and Methods. The arrow indicates the recombinant COR15m, and arrow heads indicate multimers.

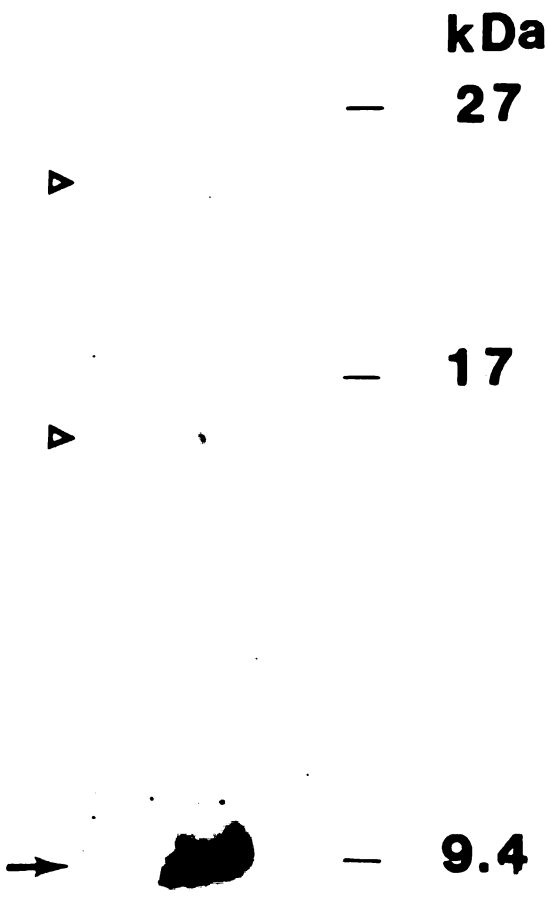

Figure 4.12

Figure 4.13. A helical wheel diagram of the putative COR15m containing 88 amino acids residues (amino acids from 51 to 139 of COR15). Each α-helix contains 3.6 residues, so the adjacent residues are separated by 100^{o} of arc on the wheel and each wheel contains 5 α-helices. The chemical properties of the amino acid side chains are shown as: +, positively charged (lys, arg); -, negatively charged (asp, glu); blank, polar (asn, gln, ser, thr); and shaded, apolar (leu, ile, val, phe, tyr, trp, ala,).

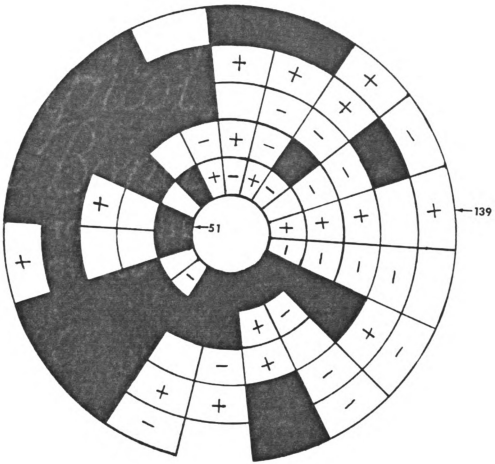

Figure 4.13

Materials and Methods), fractionated again on a tricine-SDS-PAGE gel and subjected to immunoblot analysis, multiple bands were detected at positions higher than 9.4 kDa in addition to a 9.4 kDa band (Figure 4.12). In this case, it was clear that the higher molecular weight bands could only result from the formation of multimers of the recombinant COR15m because it was the only protein in the sample. The relative migration positions of the COR15m multimers indicated that they might be dimers, and trimers (Figure 4.12).

DISCUSSION

The results of DNA sequence and immunoblot analyses indicate that the *cor15* gene of *Arabidopsis* encodes a 14.7 kDa cold-regulated polypeptide, COR15, that is processed *in planta* to a polypeptide of about 9 kDa. The mature protein, designated COR15m, is soluble, hydrophilic, and is predicted to form an α-helix. The predicted amino acid sequence of COR15m indicated that it was rich in alanine (21.1 mol%), lysine (17.8 mol%), and glutamic acid (14.4 mol%), but devoid of cysteine, proline, tryptophan, histidine, and methionine (Table 4.1). The amino acid sequence of COR15 failed to show any significant sequence homology with that of other proteins including the fish antifreeze proteins (Davies and Hew, 1990) and the plant LEA proteins (Baker *et al.*, 1988; Close *et al.*, 1989), which have been suggested to have roles in the freezing tolerance of fish and dehydration tolerance of plants, respectively. However, an analysis of the secondary structure (Schiffer and Edmunson, 1967) of COR15m revealed that it could form an amphiphilic α-helix (Figure 4.13). This amphiphilic α -helical structure has been predicted for some LEA proteins (Dure *et al.*, 1988), and has been suggested to allow for the formation of tertiary filamentous polymers by

intramolecular or intermolecular interactions (Dure *et al.*, 1988). The relationship between the suggested tertiary structure of LEA proteins and their speculated function in dehydration protection is not clear. It appears that COR15m can form intermolecular multimers (Figure 4.12B). The formation of intermolecular disulfide bound is excluded as COR15m has no cysteine residues (Figure 4.2). Whatever interaction enables the COR15m to aggregate is resistant to the detergent SDS (Figure 4.12B). Whether the formation of multimers of COR15m results in a filamentous polymer suggested for LEA proteins remains to be determined.

Immunoblot analysis indicated that COR15m is localized in chloroplasts. Specifically, COR15m can be detected in soluble protein extracts prepared from chloroplasts purified on percoll gradients (Figure 4.8). In addition, amino acid sequence comparisons indicate that the N-terminal sequence of COR15 has a number of characteristics in common with chloroplast transit peptides, including sequences that match the loose consensus stromal-targeting cleavage site (Gavel *et al.*, 1990; see Figure 4.7). The size of COR15m is consistent with COR15 being processed at the putative consensus cleavage site; the apparent molecular weight of COR15m is about 9 kDa (Figure 4.6), while the predicted molecular weight of COR15m, assuming processing at residue 50 of COR15 (the putative cleavage site) is 9.4 kDa (Table 4.1). 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 (unpublished result). However, when the putative COR15m sequence (amino acids 51-139) was fused to a methionine codon and expressed in *E. coli*, the recombinant COR15m (also boiling-stable) polypeptide comigrated on tricine -SDS-PAGE with authentic COR15m prepared from chloroplasts (Figure 4.10). Taken together, these data indicate that at least some of the cellular COR15 is transported into chloroplasts and proteolytically processed to COR15m of approximately 9 kDa.

Confirmation of this conclusion and a determination of the suborganeller location of COR15m will be accomplished by future immunolocalization studies. Based on the fact that COR15m is present in the soluble fraction of the chloroplasts and that the putative transit peptide of COR15 resembles the stroma targeting signals, the suborganeller localization of COR15m is speculated to be in the chloroplast stroma. Finally, it should be noted that the data presented here 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, Hincha, Schmitt and colleagues (Voger and Heber, 1975; Hincha *et al.*, 1989; Hincha *et al.*, 1990). 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 over 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 (Voger and Heber, 1975; Hincha *et al.*, 1989; Hincha *et al.*, 1990) indicate that they have a number of properties in common with COR15m: they are cold-regulated, hydrophilic, they remain soluble upon boiling in aqueous solution, they are small (about 10 to 30 kD) and at least some of them appear to be present in chloroplasts (see Discussion of Voger and Herber, 1975). Results presented in the previous chapter indicate that COR15 does indeed have cryoprotective activity in an *in vitro* assay. The obvious question raised is whether COR15m also has cryoprotective properties. The expression of COR15m in *E. coli* should enable the purification of COR15m in large amount and its cryoprotective activity be tested directly. The key question, of course, is whether

COR15m and the spinach and cabbage proteins have cryoprotective roles *in planta*. In particular, do they have specific functions in protecting chloroplasts against freeze -induced damage? The construction of transgenic *Arabidopsis* plants that either overexpress or underexpress *cor15*, should help in answering these basic questions.

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APPENDIX

CREATION AND ANALYSIS OF *ARABIDOPSIS* MUTANTS OVEREXPRESSING OR UNDEREXPRESSING *cor15*

I have initiated a study to create and analyze *Arabidopsis* mutants that overexpress or underexpress *cor15*, in an attempt to determine if the gene has an important role in *Arabidopsis* cold acclimation.

Transgenic *Arabidopsis* plants that constitutively express *cor15* under the control of the CaMV 35S promoter were obtained by *Agrobacterium*-mediated transformation. The transgenic plants were analyzed to determine if constitutive accumulation of COR15m resulted in increased freezing tolerance of whole plant tissues or isolated chloroplasts. The results were encouraging but inconclusive.

Attempts to create transgenic plants that underexpress *cor15* were made by transforming plants with a plasmid containing an antisense *cor15* gene under the control of the CaMV 35S promoter. More than 130 independent transformants were obtained. The immunoblot analysis of the accumulation of COR15m for approximately 100 transformants indicated that none of them dramatically underexpressed *cor15*.

I. Plasmids

A *Sca*I DNA fragment containing the whole ORF (open reading frame) for COR15 and part of the sequence from pBluescript was isolated from pLCT10A, and *Bam*HI linkers were added to the ends of the fragment that had been repaired by a standard *E. coli* DNA polymerase I reaction (Sambrook *et al.*, 1989). The DNA was then digested with *Bam*HI, and cloned into the *Bam*HI site downstream of the CaMV 35S promoter in the plasmid vector pCIB710 (Rothstein et al., 1987), and introduced into *E. coli* strain DH5α (Bamsbrook *et al.*, 1989). Clones containing recombinant plasmids with the COR15 ORF oriented in sense or antisense direction with respect to the CaMV 35S promoter in pCIB710 were isolated, and referred to as pLCT61 and 62, respectively. The *Xba*I/*Kpn*I DNA fragment of the plasmid pLCT61 (containing the sense 35S-*cor15* gene), and pLCT62 (containing the antisense 35S-*cor15* gene) were isolated, cloned into the corresponding sites of the binary vector pCIB10 that provides the T-DNA borders and the kanamycin-resistant gene *npt*II (Rothstein *et al.*, 1987), and referred to as pLCT71 and pLCT72, respectively. The plasmids pLCT71 and pLCT72 were then introduced into *Agrobacterium tumefaciens* strain LBA4404 (Hoekema *et al.*, 1983) by triparental mating with pRK2073 providing the mobilization functions.

II. *Arabidopsis* Transformation

Arabidopsis thaliana ecotype RLD was transformed by the root explant cocultivation and regeneration method either exactly following the procedure of Valvekens (Valvekens *et al.*, 1988) or with the slight modifications as described bellow.

A. Preparation of *Arabidopsis* root explants and cocultivation

Arabidopsis seeds were surface sterilized by incubating seeds (about 100 mg) in 1.2 ml of LD^{TM} (Alcide Co., Norwalk, CT) disinfectant solution (water:base:activator = 10:1:1) at 30^{o}C for 30 min. The seeds were then washed 3 times with sterile water, suspended in sterile water, and spread as straight lines on 150 x 25 mm Petri dishes containing germination medium (GM) (Valvenkens *et al.*, 1988) supplemented with B_5 vitamins (0.1 mg/l thiamine, 0.01 mg/l nicotinic acid, 0.01 mg/l pyridoxine) and 1 mg/l myo-inositol. About 1 mg seed was spread as one line on the surface of one GM plate. The sterilizing effect of LD^{TM} solution was found to be better than the standard ethanol/NaOCl method (Valvenkens *et al.*, 1988). The plates were sealed with two layers of parafilm (American National Can, CT), and placed at a 45^{o} to 60^{o} angle under white fluorescent lights so that the roots would only grow on the surface of the agar medium but not penetrate into the agar. The light intensity was about 100 μE s^{-1} m^{-2} , the photoperiod 16 h, and the temperatures ranged from 23^{o}C to 27^{o}C. After 3 weeks, plantlets were cut with a razor blade to remove the green parts, the roots were peeled off from surface of the agar medium with forceps and placed onto Petri dishes containing callus inducing medium (CIM) (Valvenkens *et al.*, 1988) supplemented with B_5 vitamins. The intact roots were incubated on CIM for 3 days (never in excess of 4 days to avoid a dramatic decrease in the transformation efficiency), and then transferred to a clean Petri dish, cut to small pieces of 0.5 to 1 cm long, and incubated with *Agrobacterium* cells for 3 min. The *Agrobcterium* cells harboring the plasmids (pCIB10, pLCT71, or pLCT72) were grown in LB (0.5% NaCl, 0.5% yeast extract, 1% Bacto-tryptone) supplemented with 50 mg/l rifampicin and 50 mg /l kanamycin, at 30^{o}C for 24 to 48 h. The bacterial cells were washed twice with fresh LB containing no antibiotics, resuspended in LB, and mixed with the root explants. The root explants

were then transferred onto CIM plates, incubated for 2 to 3 days, the root explants were collected, washed at least 3 times by placing them in bottles containing more than 150 ml of sterile water, shaking vigorously for 1 to 2 min, changing the water and washing again until the water was clear after shaking.

B. Shoot induction, root induction, and seed production

The root explants were placed onto the shoot inducing medium (SIM) (Valvenkens *et al.*, 1988) supplemented with B_5 vitamins, with a few pieces of roots clumped together. The antibiotics vancomycin (500 mg/l) or carbenicillin (500 mg/l) was used to inhibit the growth of *Agrobacterium*; carbenicillin was found to be much more effective than vancomycin for this purpose. Green calli appeared in about 10 days, and they developed into shoot rosettes in another 1 to 2 weeks. The calli or small shoots were transferred to fresh SIM plates every week until the shoots were big enough for root induction. When the shoots were big enough (1-2 weeks from calli), they were individually excised from the calli but with a small chunk of the callus attached to the bottom of the shoot, incubated about 2 to 5 min in a drop of root inducing hormone solution (1 mg/ml indole-3-butric acid [sodium salt], 2 mg/ml 1 -naphtaleneacetamide in water) (Last *et al.*, 1991), and placed in a magenta box containing GM medium. A hole (about 2.5 cm in diameter) was made the in lids of the magenta boxes and the hole was plugged with a polyurethane foam plug (Apico Inc., Baltimore, MD) to accelerate air exchange and prevent contamination. Roots appeared within one week. After about 2 to 3 weeks grown on GM medium, plantlets were pulled out from the medium, all agar surrounding the roots was removed by washing with water, the plantlets were transferred to soil, and covered with plastic wrap. These transgenic plants (referred to as T_1 plants) were grown in growth chambers under the conditions described in Chapter 2, the plastic wrap covering the pots was cut to

decrease the humidity one week after transfer and removed another week later. Seeds were harvested from individual plants about 4 weeks after planting in soil, and referred to as T_2 seeds. Seeds from individual T_2 plants were harvested and referred to as T_3 progeny.

III. Analysis of transgenic plants

A. Kanamycin assay

Transformed plants were evaluated initially as those able to grow normally on GM medium containing kanamycin. The seeds were surface sterilized as described previously, and germinated on Petri dishes containing GM medium supplemented with 75 mg/l kanamycin. The germinating transformants grew normally, but the untransformed plantlets became yellowish within a week, became bleached and died within 10 days. The surviving plantlets were transferred to soil after two weeks on GM plates, and the transplants were covered with plastic wrap. The plastic wrap was removed after 3 days, and the plants were allowed to grow and set seed.

Segregation of kanamycin-resistant and sensitive individuals was scored for T_2 plants, and summarized in Table A.1. The transgenic lines CIB-1, 71-2, and 71-3 segregated in a 3:1 ratio for kanamycin resistant to sensitive individuals (Table A.1).

B. Detection of *cor15* sense and antisense transcript in transgenic plants

Total RNA was extracted from wild type RLD plants and transgenic plants as described in the Chapter 2. RNA (15 μg per lane) was resuspended in formaldehyde loading buffer containing EtBr (1 mg/ml), and fractionated on denaturing formaldehyde agarose gels (Sambrook *et al.*, 1989). Northern blots were prepared on Nytran membranes

Table A.1 Analysis of kanamycin resistance of the T_2 progeny for some of the transgenic plants

Progeny	Kan^r	Kan^s	Kan^r/Kan^s	Plasmid transformed
CIB-1	10	3	3.3:1	pCIB10 (vector)
71-1	5	0	5:0 *	pLCT71 (sense *cor15*)
71-2	15	8	2.5:1	,,
71-3	10	4	3.3:1	,,
72-31	30	13	2.3:1	pLCT72 (antisense *cor15*)
72-33	1	90	1:90 *	,,
72-34	75	14	5.3:1	,,
72-35	19	7	3.7:1	,,
72-36	107	12	8.9:1 *	,,
72-37	130	37	2.8:1	,,
72-38	0	37	0:37 *	,,
72-39	40	8	4.8:1	,,
72-40	85	28	3.0:1	,,
72-41	86	27	3.2:1	,,
72-42	78	6	13 :1 *	,,
72-43	51	31	1.6:1 *	,,
72-44	15	26	0.5:1 *	,,
72-46	25	5	5.0:1	,,
72-47	4	2	2.0:1	,,
72-48	56	17	3.3:1	,,
72-49	36	6	6.0:1	,,
72-52	46	16	2.9:1	,,
72-55	26	14	1.9:1	,,
72-59	32	2	16 :1 *	,,
72-60	2	2	1.0:1	,,
72-63	32	0	32 :0 *	,,
72-64	27	12	2.3:1	,,

* The asterisks indicate that the segregation differ significantly from a 3:1 ratio [kanamycin resistant (kan^r):kanamycin sensitive (Kan^s)] by a Chi-square test ($P_{0.1}$).

(Schleicher and Schuell) using 20 x SSPE (Sambrook *et al.*, 1989). The blotted RNA was visualized and photographed with UV irradiation, blots baked at 80°C for 3 h in a vacuum oven, and then washed in 2 x SSPE containing 0.5% SDS at 55°C for 3 h. Sense and antisense *cor15* RNA probes (5-7 x 10^5 cpm) were prepared by *in vitro* transcription of the plasmid pLCT10B and pLCT10A, respectively. The plasmid DNAs (0.2 μg) digested with *Bam HI* were transcribed in 20μl reactions (containing 40 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 1 unit RNAsein, 500 μM each of ATP, GTP, and CTP, 12 μM UTP, 250 μCi [^{32}P]UTP, and 25 units of T_7 RNA polymerase) at 37°C for 1 h. The reactions were then mixed with 10 unit of DNAse I, incubated at 37°C for 30 min, diluted with 100 μl of TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA), heated at 65°C for 5 min, chilled in ice, and the RNAs purified using a G-75 Sephadex spun column (Sambrook *et al.*, 1989). Hybridization was carried out according to standard procedures (Sambrook *et al.*, 1989) at 42°C for 16 h, the blots were then washed twice with 0.1 x SSPE containing 0.5% SDS, incubated (without shaking) in 0.1 x SSPE containing 0.5% SDS at 60°C for 48 h, wrapped in a plastic membrane, and exposed to X-ray films.

cor15 transcripts were detected in T_3 plants (71-1-3, 71-2-23, 71-3-1) of all three transgenic lines transformed with the sense 35S-*cor15* gene (Figure A.1). The transcripts of the transformed gene was slightly larger than that of the native ones (Figure A.1), and were expressed at lower levels in comparison to that of the native *cor15* transcripts (Figure A.1). 71-1-3 and 71-2-23 are T_3 progeny (of the lines 71-1 and 71-2, respectively) that were homozygous for kanamycin resistance, while 71-3-1 plants (T_3 progeny of the line 71-3) segregated in a 3:1 ratio for kanamycin-resistant to sensitive individuals.

Figure A.1. Nothern blot analysis showing the *cor15* transcript in nonacclimated (W) and cold-acclimated (C) plants of wild type (RLD), and "sense" transgenic lines 71-1-3 (71-1), 71-2-23 (71-2), and 71-3-2 (71-3). The blot was hybridized with an antisense RNA probe for *cor15* prepared by *in vitro* transcription. Exposure was overnight.

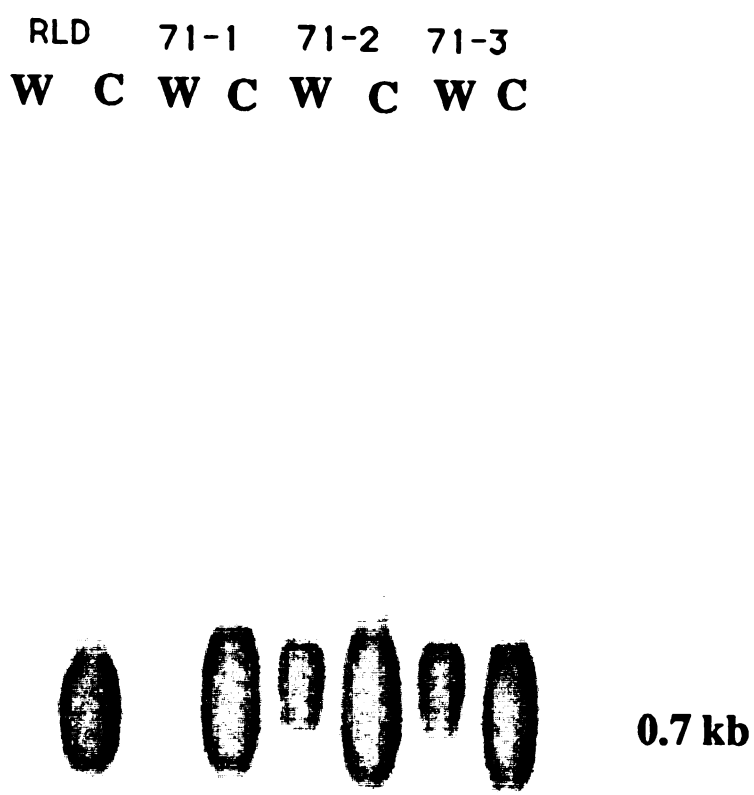

Figure A.1

Northern blot analysis demonstrated the presence of the antisense-*cor15* RNA in T_2 plant of all three randomly chosen transgenic lines that were transformed with the antisense 35S-*cor15* gene (Figure A.2). The levels of RNA of the native *cor15* gene in these lines, however, was not dramatically affected by the presence of the antisense RNA (Figure A.3). This is not necessarily surprising as the line producing the most antisense, 72-2, only produced only about 1/2 to 1/4 the amount of RNA (*cor15* antisense) as was produced by the endogenous *cor15* gene.

C. Detection of COR15m in the transgenic plants

Total soluble protein was isolated from wild type RLD and transgenic plants, proteins were fractionated on SDS-PAGE gels, and immunoblots analyzed with antibodies against COR15 as described in Chapter 4.

Immunoblot analysis indicated that the sense transgenic plant accumulated COR15m constitutively. The levels of accumulation of COR15m in nonacclimated T_3 plants 71-2-23 and 71-3-1 (sense transgenic lines 71-2 and 71-3, respectively) were found to be comparable to the level in the cold-acclimated wild type plants (Figure A.4). The levels of COR15m in cold-acclimated 71-2-23 and 71-3-1 plants appeared to be a bit higher than that the cold-acclimated wild type plants (Figure A.4). The COR15m synthesized in the nonacclimated transgenic plants was targeted to the chloroplasts (not shown). However, the immunoblot analysis of the antisense transgenic lines (kanamycin resistant T_2 plants) indicated that none of these lines dramatically decreased its COR15m level (not shown).

D. CO_2-dependent oxygen evolution assay

The effect of constitutive accumulation of COR15m on the freezing tolerance of plants was assessed using oxygen evolution assays. Light-dependent oxygen evolution

Figure A.2. Nothern blot analysis showing the antisense RNA of *cor15* in nonacclimated (W) and cold-acclimated (C) plants of wild type (RLD) and "antisense" transgenic lines 72-2A, 72-2B, and 72-20. The blot was hybridized with a sense RNA probe for *cor15* prepared by *in vitro* transcription. Exposure was two days.

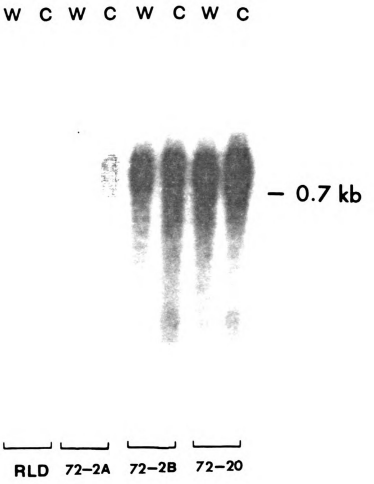

Figure A.2

Figure A.3. Nothern blot analysis showing the *cor15* transcript in nonacclimated (W) and cold-acclimated (C) plants of wild type (RLD) and antisense transgenic lines 72-2A, 72-2B, and 72-20. The blot was hybridized with the antisense RNA probe for *cor15* prepared by *in vitro* transcription.Eexposure was overnight.

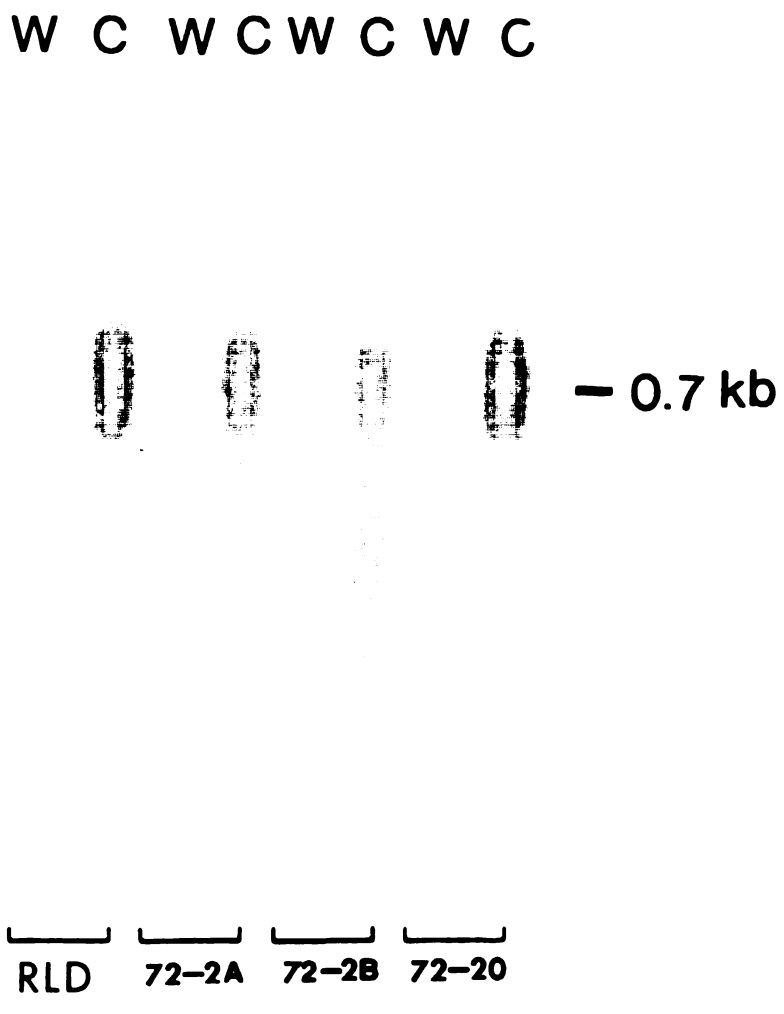

Figure A.3

Figure A.4. Immunoblot analysis showing the accumulation of COR15m in nonacclimated (W) and cold-acclimated (C) plants of wild type (RLD) and sense transgenic lines 71-1-3, and 71-2-23. Total soluble protein was fractionated by SDS-PAGE on a 15% gel, transferred to a nitrocellulose, and the blot developed with antiserum (1:25 dilution) against COR15-protein A fussion protein (see Chapter 4).

Figure A.4

activity reflects the integrity of the chloroplast electron transport apparatus. *Arabidopsis* RLD and T_3 transgenic plants 71-2-23 were grown with or without cold acclimation under the conditions described in Chapter 4. O_2 evolution assays were conducted with leaf discs according to Walker (Walker, 1985), using a Clark type oxygen electrode unit (Runk Brothers, Cambridge, England). Leaf discs (approximately 1 cm in diameter) were obtained using a paper hole puncher. Each sample, consisting of 10 leaf discs, was equilibrated at -2°C in glass test tubes for 30 min in a low temperature bath (Model 2095, Forma Sci., Ohio). Freezing was then initiated by the addition of a drop of fine ice onto the leaf discs, and the temperature was decreased at a rate of 1°C/30min. After being frozen at appropriate temperatures for 30 min, tubes were transferred to ice, and kept on ice in the dark overnight to allow the leaf discs to thaw slowly. Leaf discs were cut into 4 strips each with a razor blade, immersed in the assay solution (containing 100 mM tricine-KOH, pH8, 50 mM KCl, 5 mM $NaHCO_3$) that was saturated with N_2 and maintained at 21°C with a circulating water bath, and equilibrated in the dark for 2 min. The oxygen evolution reaction was started by illumination with white light of approximately 300 $\mu E\ m^{-2}\ s^{-1}$, and the rate of oxygen evolution measured for 5 to 10 min. The plant material was recovered, blotted dry on filter paper, incubated in 1 ml acetone in an eppendorf microtube at room temperature for 1 h, and the content of chlorophyll measured at OD_{652} (μg chlorophyll = 2.78 OD_{652}). The rate of oxygen evolution for freezing-treated samples was expressed as a percentage of the rate of untreated samples (approximately 50 to 100 $\mu M\ O_2\ mg\ chl^{-1}\ h^{-1}$).

Figure A.5 and A.6 demonstrate that freeze/thaw treatment severely damaged the ability of plant tissues to evolve oxygen for both nonacclimated wild type plants and 71-2-23 plants (Figure A.5). Cold acclimation increased the resistance of the photosynthetic apparatus in both kinds of plants to freeze inactivation (Figure A.6).

Freezing leaves at -7°C almost completely abolished the oxygen evolution activity of both nonacclimated wild type and 71-2-23 plants (Figure A.5). Freezing at this temperature, however, had little effect on the cold-acclimated plants (Figure A.6). In one experiment, leaves from 71-2-23 plants were more resistant to freezing damage than those from the wild type plants (Figure A.5a), but this increased freezing tolerance of 71-2-23 plants was not observed in another experiment (Figure A.5b) where a different batch of wild type and 71-2-23 plants were used. Similar inconsistencies in the experimental results were found when cold acclimated plants were used (Figure A.6). The reason for these inconsistencies is unknown. It may result from deviations derived from experimental conditions (e.g. time to prepare leaf discs, time to thaw leaf discs, etc) that were not precisely controlled, or it could because of some subtle differences in the growth conditions of the plants (e.g. temperature, light intensity, humidity, time the plant materials harvested, etc).

E. Photophosphorylation

The effect of constitutive accumulation of COR15m on the cryobehavior of the chloroplasts was analyzed directly by measurement of the photophosphorylation activities of the chloroplasts after a freeze/thaw cycle or after an electrolyte (NaCl) treatment. The effect of freeze/thaw cycle and high concentration of NaCl on the photophosphorylation activity for chloroplasts isolated from the sense transgenic line 71-3-5 (homozygous for kanamycin resistance, and constitutively accumulates COR15m), and a quasi wild type line 71-3-3 (homozygous for kanamycin sensitivity, and does not accumulate COR15m until cold acclimated), were compared.

Chloroplasts were isolated essentially as described in Chapter 4 but without the step of Percoll gradient centrifugation; a different grinding buffer (0.45 M sorbitol, 20 mM Hepes-KOH pH 7.8, and 5 mM EDTA), and a slightly different resuspension

Figure A.5a. Oxygen evolution of leaf discs prepared from nonacclimated wild type RLD plants (broken line) and the sense transgenic line 71-2-23 (solid line). Leaf discs were treated with a freeze/thaw cycle at the temperatures indicated proir to the assay. The oxygen evolution activity of each sample was expressed as percentage of that of the untreated sample that was kept on ice.

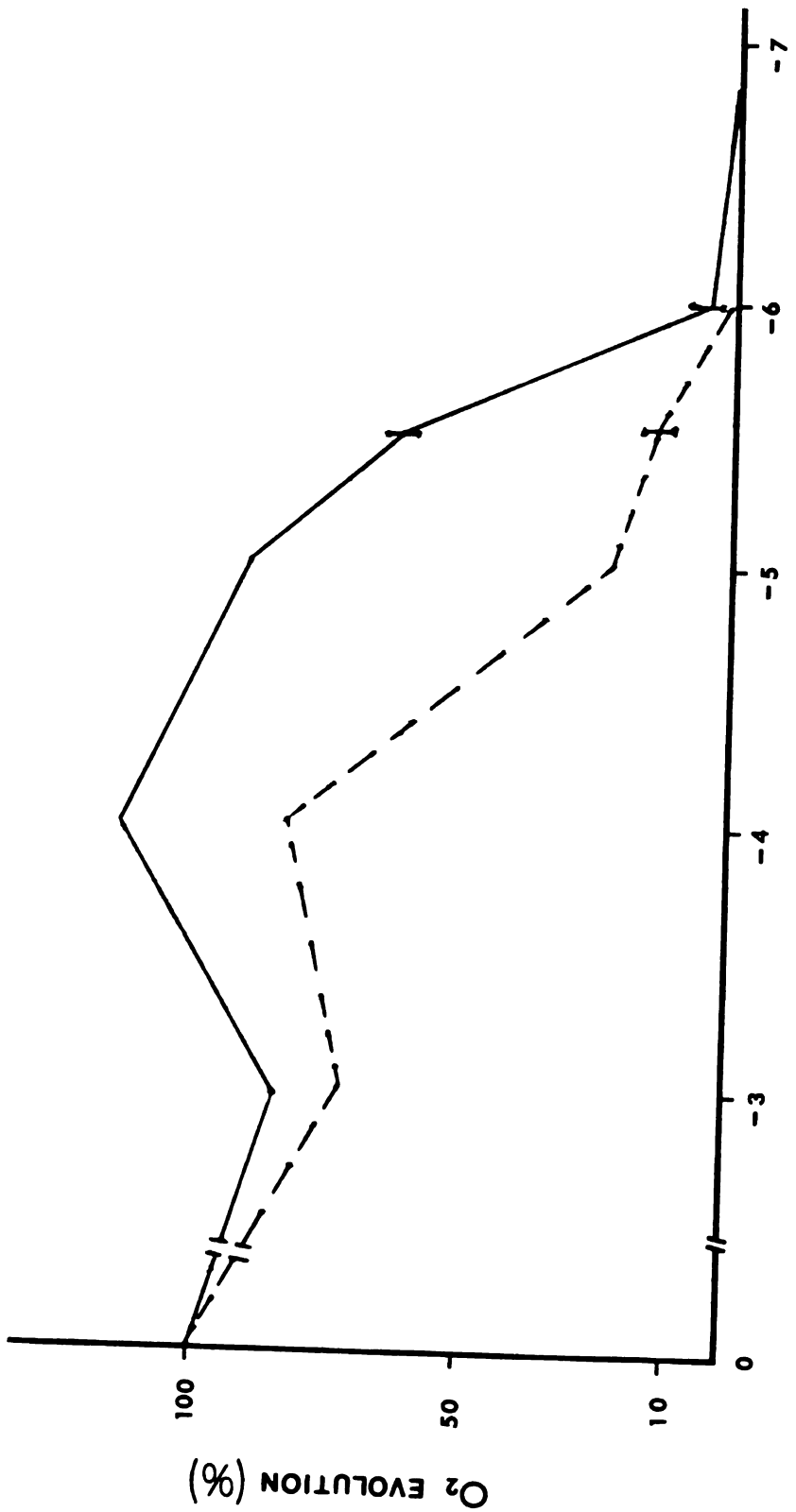

Figure A.5a

Figure A.5b. Same as Figure A.5a using a different batch of plants.

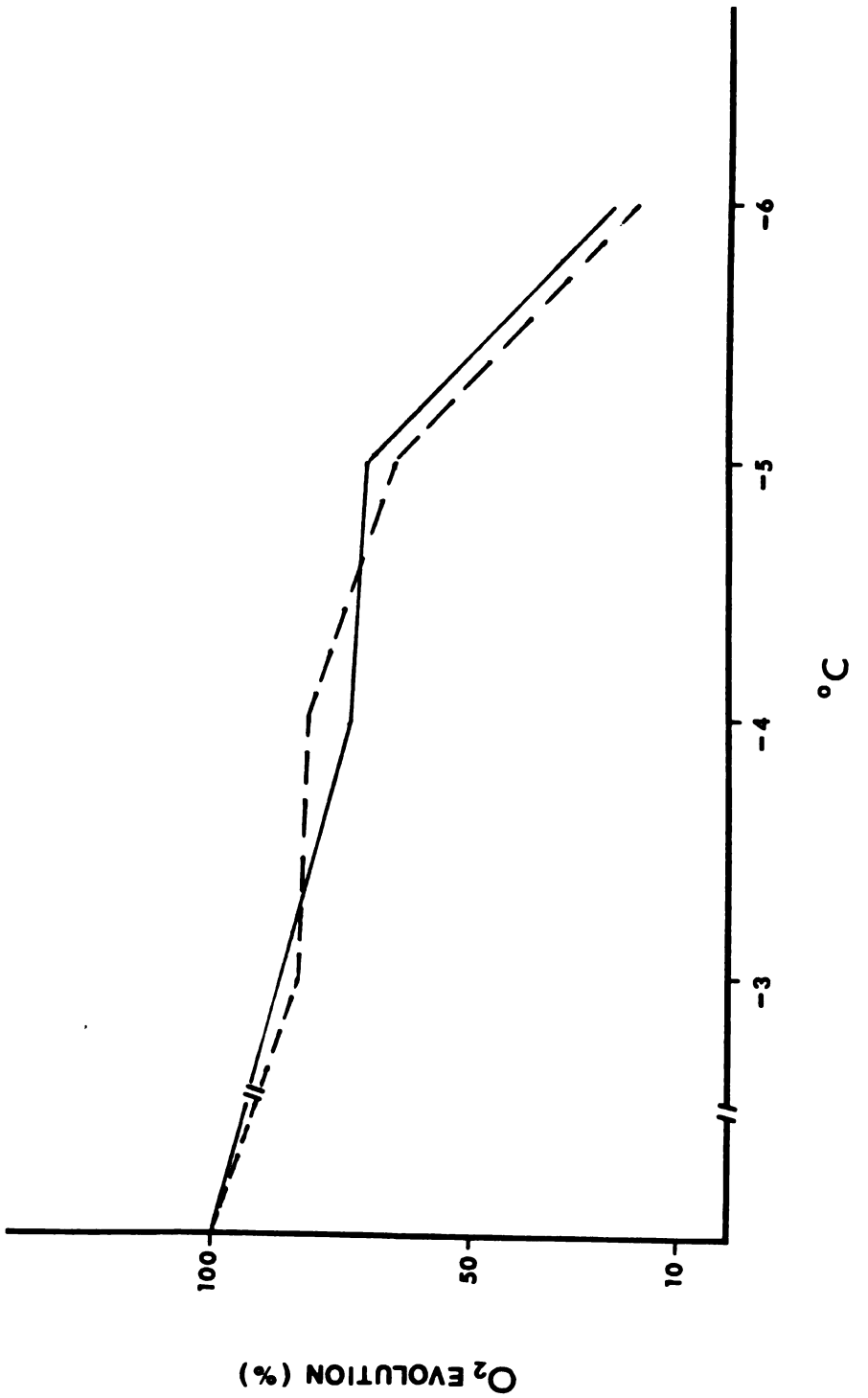

Figure A.5b

Figure A.6a. Oxygen evolution analysis of leaf discs prepared from cold-acclimated plants of wild type RLD (broken line) and the sense transgenic line 71-2-23 (solid line). Leaf discs were treated with a freeze/thaw cycle at the temperatures indicated proir to the assay. The oxygen evolution activity of each sample was expressed as percentage of that of the untreated sample that was kept on ice.

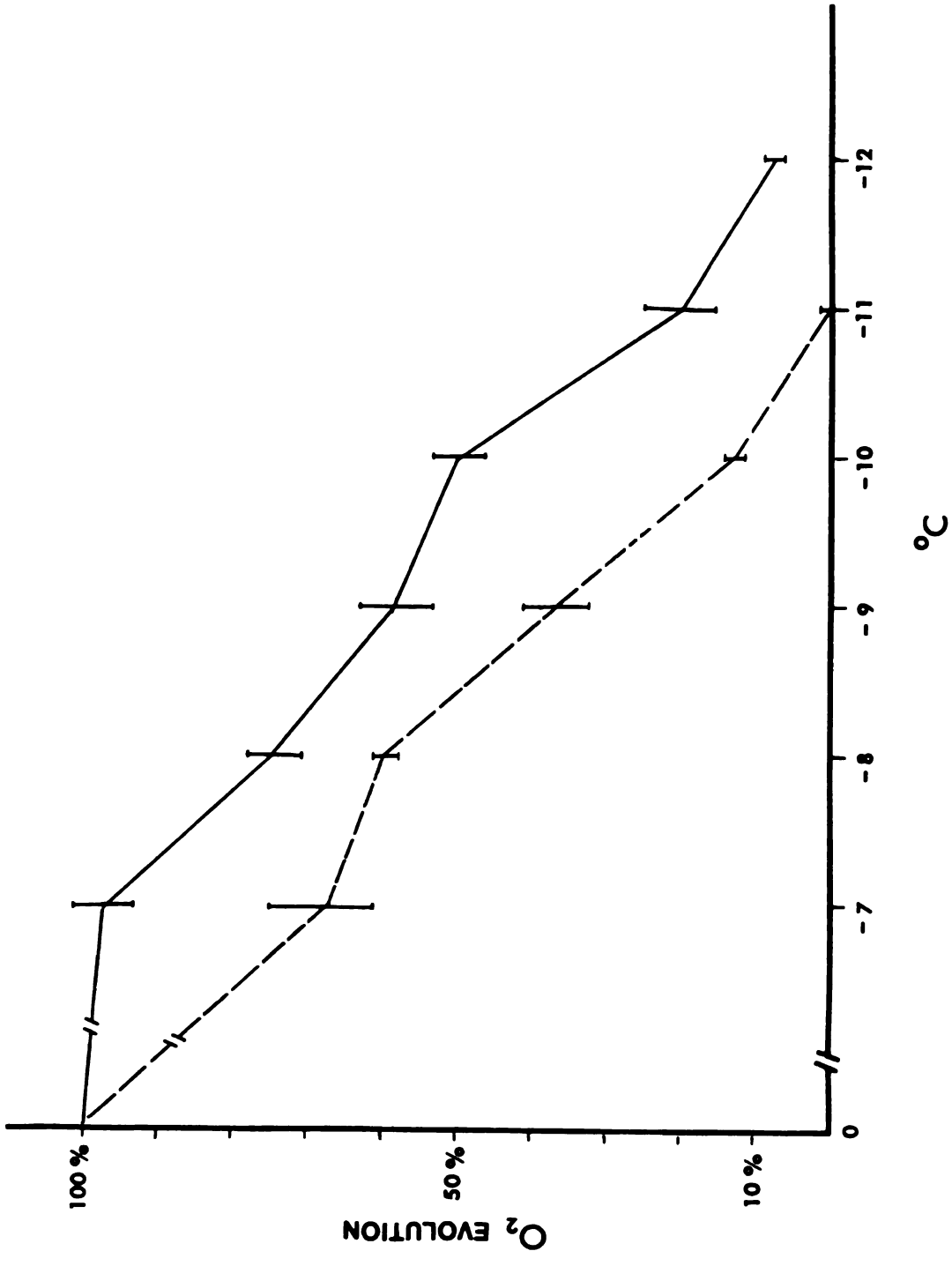

Figure A.6a

Figure A.6b. Same as Figure A.6a using a different batch of plants.

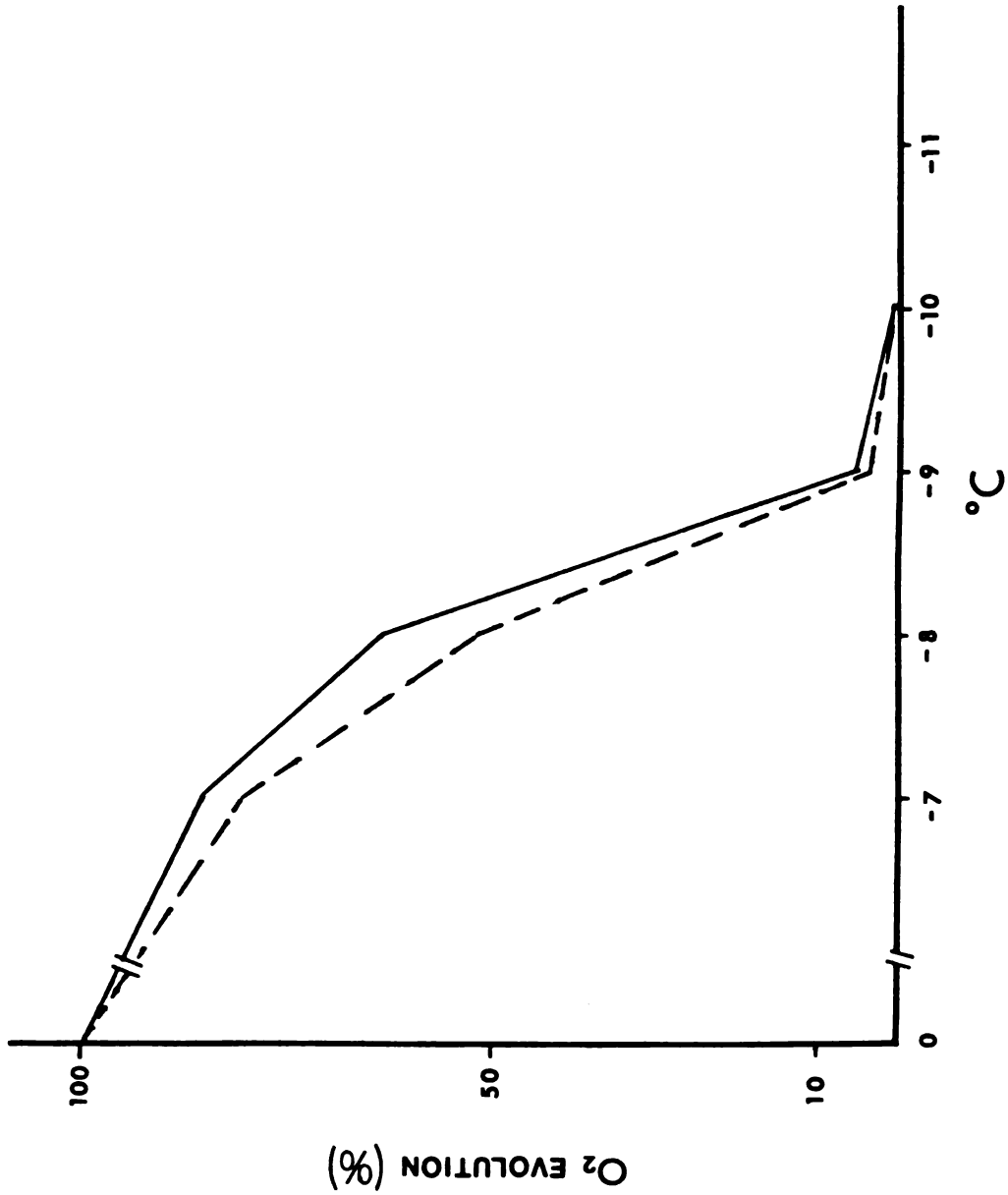

Figure A.6b

buffer (0.33 M sorbitol, 20 mM Hepes-KOH pH 7.8, and 5 mM $MgCl_2$) were used. Chloroplasts obtained from the first centrifugation were resuspended in theresuspension buffer to 2 μg chl/μl and stored on ice in the dark. The intactness of the chloroplasts was checked by microscopy, and by ferricyanide-dependent oxygen evolution (Leegood and Malkin, 1986), and the chloroplasts were classified as type B (unbroken) according to the criteria described by Reeves and Hall (Reeves and Hall, 1980). For the freezing treatment, 100 μl of the chloroplast suspension (0.5 μg chl/μl) was added to a glass test tube, and incubated at -2^{o}C for 10 min. Freezing was initiated by the addition of a few grains of sand cooled to -20^{o}C, and the temperature decreased at a rate of 1^{o}C/30 min. The samples were then thawed on ice for 2 to 3 h before the photophosphorylation assay. For the NaCl treatment, chloroplast suspensions were made of 0.5 μg chl/μl and 0.2 to 2 M NaCl in microcentrifuge tubes, and were incubated on ice for 3 h before the assay. Photophosphorylation of the chloroplasts was measured as described by Mills (Mills, 1986). Briefly, 60 μl of the chloroplast suspension (0.5 μg chl/μl) was mixed with 800 μl reaction solution (70 mM tricine-KOH pH 8, 50 mM glucose, 30 mM KCl, 12 mM $MgCl_2$, 1 mM NaH_2PO_4, 2 mM ADP, 0.1 mM Methylviologen, 2.5 μM diadenosinepentaphosphate, 4 unit/ml hexokinase, 5 μCi/ml ^{32}Pi) in an microcentrifuge tube, allowed to equilibrate in the dark for 2 min, and illuminated with white light (approximately 200 μE m^{-2} s^{-1}) for 3 min. The unincorporated ^{32}Pi was extracted with molybdate (Mills, 1986), and the amount of ^{32}Pi incorporated into ATP (approximately 10^5 cpm for the untreated controls) was measured in a liquid scintillation counter. The photophosphorylation reaction was completely inhibited by the addition of 20 mM DCMU (electron transport inhibitor), or 5 mM NH_4Cl (uncoupler), or by omission of the light. The relative photophosphorylation activity of the freezing- or NaCl-treated samples was expressed as percentages of that of the untreated sample.

It is intriguing to find that the chloroplasts isolated from 71-3-5 plants that accumulat*ed* COR15m constitutively, had a slightly higher degree of freezing tolerance than those of the quasi wild type (71-3-3) plants (Figure A.7). Similar results were obtained when the chloroplasts isolated from a second batch of plants were analyzed (not shown). More experiments are needed, however, to comfirm these results.

At temperatures occurring in nature, intracellular freezing is considered to be universally lethal to the plant cell, so freezing resistance can be more accurately defined as a tolerance to the consequences of extracellular freezing. One of the most detrimental consequences of extracellular freezing is dehydration of the protoplasm and concentration of the electrolytes to toxic levels. An analysis of the cryobehavior of isolated chloroplasts upon a direct freeze/thaw cycle, therefore, may not be the best way as chloroplasts may never freeze in plant cells surviving a freezing stress in nature. Thus, an alternative method to detect the effect of COR15m on chloroplast cryobehavior was employed by measuring the photophosphorylation activity of chloroplasts exposed to high concentrations of the electrolyte NaCl at low nonfreezing temperature (Figure A.8). As shown in Figure A.8, photophosphorylation activity of chloroplast decreased dramatically when the concentration of NaCl increased from 0 to 1.5 M. It is interesting that chloroplasts isolated from the sense transgenic plants (71-3-5) retained more than 90% of its photophosphorylation activity after incubating in 0.2 M NaCl while chloroplasts isolated from the quasi wild type plants (71-3-3) lost about 50% of its activity (Figure A.8a). However, this observation was not consistant under the experimental conditions used, as chloroplasts that were isolated from a different batch of plants of the lines 71-3-3 and 71-3-5, did not respond differentially to NaCl treatment (Figure A.8b).

Figure A.7. Photophosphorylation assay showing the response of chloroplasts to a freeze/thaw cycle. The chloroplasts were isolated from nonacclimated plants of the sense transgenic line 71-3-5 (solid line) or the quasi wild type line 71-3-3 (broken line). The photophosphorylation activity of the sample was expressed as a percentage of that of the untreated sample stored on ice.

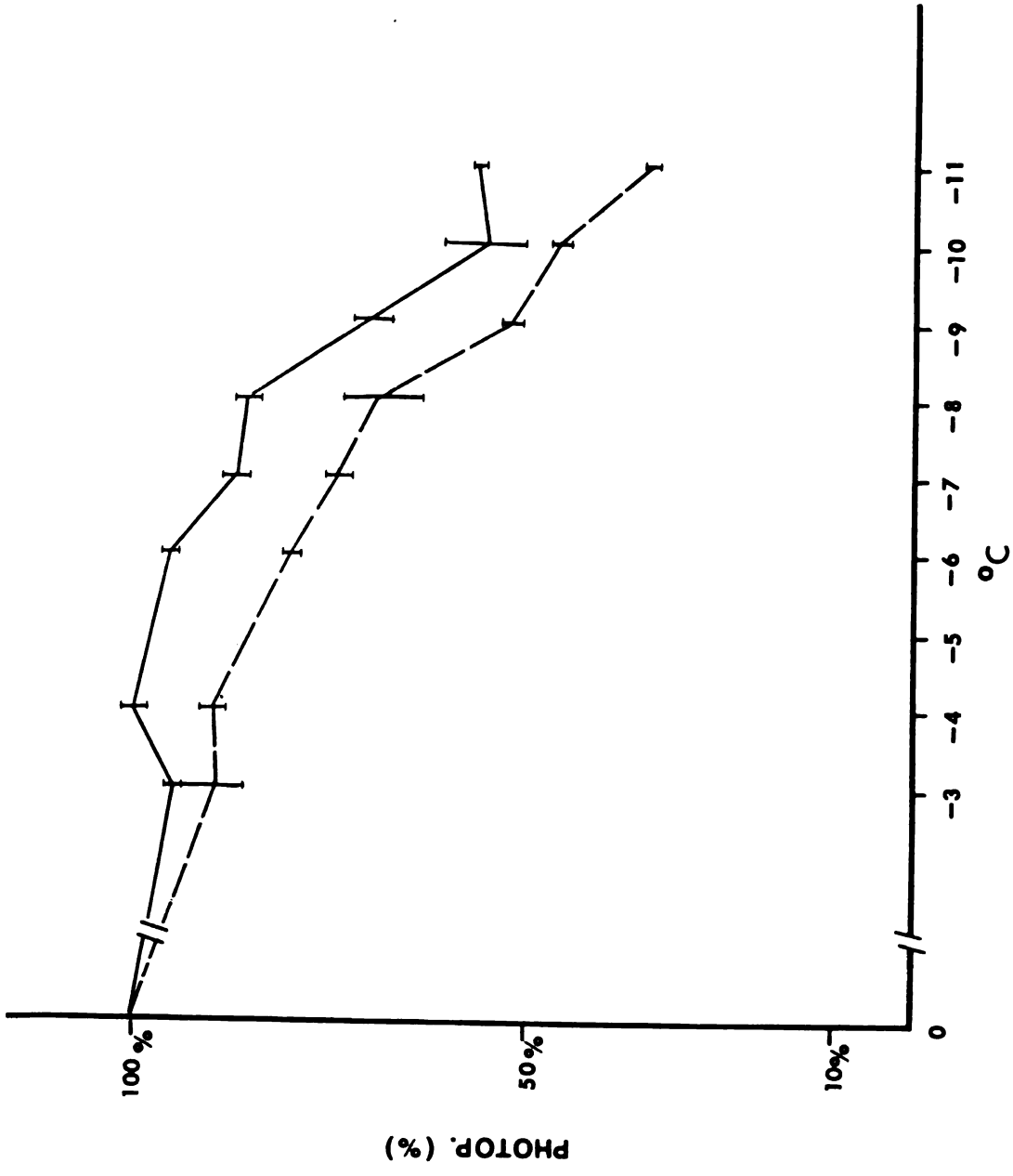

Figure A.7

Figure A.8a. Photophosphorylation assay showing the response of chloroplast to different concentrations of NaCl. The chloroplasts were isolated from nonacclimated plants of the sense transgenic line 71-3-5 (solid line) and the quasi wild type line 71-3-3 (broken line). The photophosphorylation activity of the sample was expressed as a percentage of that of the untreated sample stored on ice.

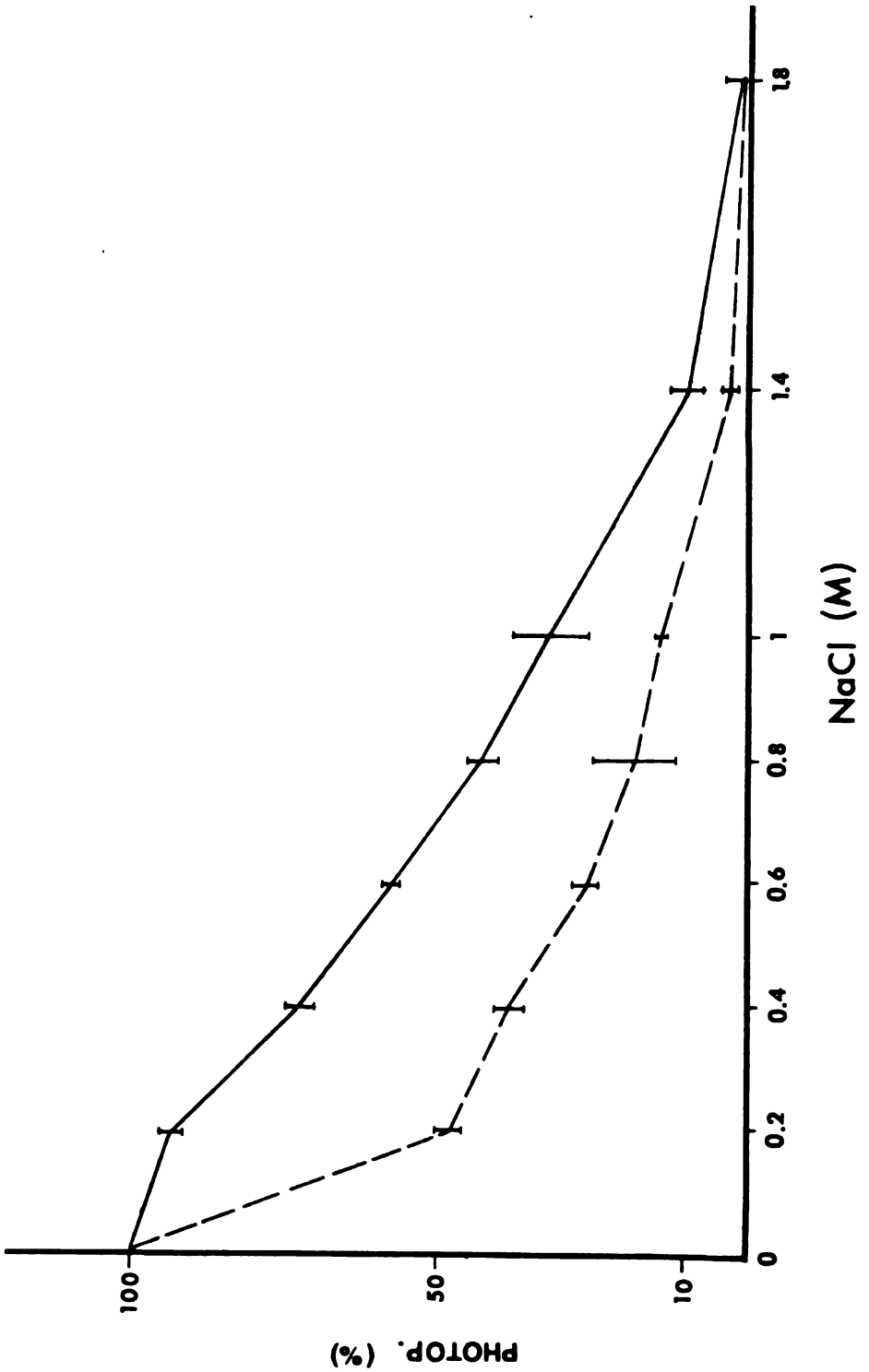

Figure A.8a

Figure A.8b. Same as Figure A.8a using a different batch of plants.

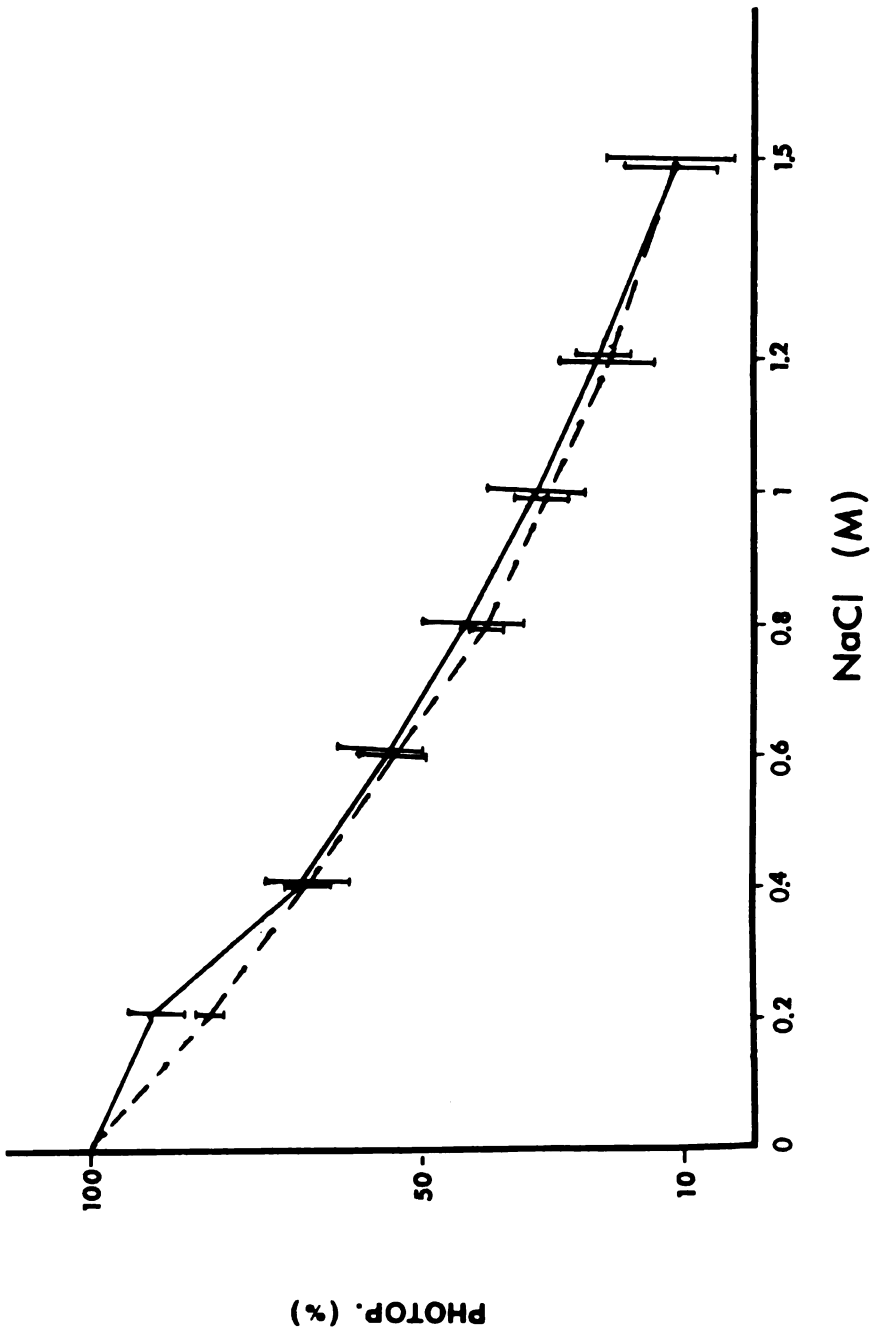

Figure A.8b

IV. Conclusion

Attempts were made to study the effect of the constitutive accumulation of COR15m on the photosynthetic functions of the chloroplasts after a direct freeze/thaw cycle or after a treatment of a high concentration of NaCl. The studies, however, were inconclusive as to whether the accumulation of COR15m in nonacclimated plant cells or its accumulation at relatively higher levels in the cold-acclimated cells could increase the resistance of the chloroplasts to freeze/thaw cycle or to high concentrations of NaCl. The inconsistency in the experimental results may be as a result of the experimental conditions not being controlled precisely enough. On the other hand, accumulation of COR15m by itself, may not result any notable differences in the cryobehavior of plant tissue or chloroplasts, as COR15m, even if necessary, may not be sufficient to protect chloroplasts from freeze inactivation. Therefore, construction of mutant plants that underexpress *cor15* may be a better approach to assess the role of COR15m in cold acclimation.

Attempts to create antisense mutants of *cor15* resulted in many transgenic plants. However, none of these transgenic lines tested show a dramatic decrease in the level of COR15m during cold acclimation.

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SUMMARY AND PERSPECTIVES

The study described in this dissertation represents an attempt to understand possible function(s) of the cold regulated (*cor*) gene in plant cold acclimation and freezing tolerance. It is demonstrated that the accumulation of transcripts encoding boiling-stable polypeptides is a common response to low temperature in different plant species. It is hypothesized that *Arabidopsis cor* genes encoding boiling-stable polypeptides have important function(s) in the plant cold acclimation and freezing tolerance. One possible role of the boiling-stable COR polypeptides is proposed to be cryoprotectant: a molecule which can protect cellular structures and macromolecules against freeze or conditions associated with freeze, such as dehydration, extreme pH, and extreme ionic strength. In support of this hypothesis, one of the boiling-stable COR polypeptides, COR15, is shown to be a potent cryoprotectant in an *in vitro* assay to protect LDH from freezing inactivation. It is not known, however, if COR15 has cryoprotective activity *in vivo*.

COR15 is found to be transported to chloroplast and processed to a mature form (COR15m) of 9.4 kDa. COR15m is also boiling-stable, and it is a hydrophilic polypeptide with an acidic pI and unusual amino acid composition. More interestingly, COR15m is predicted to have an amphapathic α-helix structure, and it is found to form multimers even in the presence of SDS. A working hypothesis is that COR15m contributes to plant freezing tolerance by acting as a cryoprotectant in chloroplast, it protects chloroplast enzymes or membranes from freeze-induced dehydration damage. For example, COR15m may protect chloroplast enzymes from dehydration-induced

injuries. On the other hand, COR15m may prevent chloroplast membranes from the occurrence of lamellar-to-hexagonal II phase transition during freezing-induced dehydration, so that the osmotic responsive nature of the membrane can be protected. It is estimated that the concentration of COR15m in chloroplast is at μM range in comparison to the CP_{50} of COR15 that is only at nM range [the calculation of COR15m concentration is based on a measurement that the yield of COR15m extracted from 12 gram leave tissue is approximately 1 μg (not shown), and a speculative estimation that chloroplasts consist of approximately 10% of the tissue volume]. It may suggest that the concentration of COR15m is at a physiologically meaningful level as its hypothesized function is concerned.

To test the working hypothesis, there are at least three approaches that can be employed. First, a gene expressing COR15m has been created and expressed in *E. coli* so that the purification of COR15m in large quantity is possible. The purified COR15m can be tested *in vitro* to see if it can protect the chloroplast enzyme from freeze- or dehydration-induced inactivation, or if it can protect the chloroplast membrane from dehydration-induced lamellelar-to hexagonal II phase transition. Secondly, the function of COR15m can be tested *in vivo* in the *Arabidopsis* mutants that constitutively express *cor15*. This stratage is basically trying to answer the similar questions as does the first approach but with less artificial conditions used in the analysis. The analysis of such mutants has yield some interesting results (see Appendix), but more experiments are needed before a conclusion can be reached. Finally, the most direct approach to test the hypothesis is to creat and analyze the *Arabidopsis* mutants that do not express *cor15* gene. Initial attempts to creat such a mutant was not successful (see Appendix). This might be because of the following reasons. First, the promoter (CaMV 35S) used to control the antisense *cor15* gene may not be capable of producing a sufficient amount of the antisense RNA to eliminate the

cor15 RNA; employment of the promoter of *cor15* gene in the antisense gene construct should solve this possible problem. Secondly, the antisense RNA produced by the 35S-antisense-*cor15* gene (see Appendix) may have secondary structures that interfer with its function; transgenic plants transformed with new antisense genes created by using the different part of the *cor15* sequences may be useful tests for this possibility. Finally, it is known that the expression of *cor15* is also regulated by dehydration stress and its expression may be necessary for plant dehydration tolerance; since the transgenic plants regenerated *via* the tissue culture method may need to survive dehydration stress at various stages of the regeneration, a null mutant of *cor15* may be selected against. Construction of a new antisense gene under the control of a regulated promoter (eg. substituted benzensulfonamide compound-induced promoter In 2-1 or In 2-2) may allow the recovery of the *cor15* deficient mutants. Alternatively, a "directe transformation" procedure without involvement of the tissue culture may overcome the same problem without change of the promoter.