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ABSTRACT

MOLECULAR STUDY OF COLD ACCLIMATION IN WHEAT

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Previous studies have shown that changes in gene expression occur in wheat during cold acclimation. Here, I show that at least some of these changes are at the RNA level. Further, some of the cold-induced mRNAs encode polypeptides which have the unusual property of remaining soluble upon boiling, a property found in certain cold-induced polypeptides of *Arabidopsis*. By cDNA cloning, and Southern and Northern analysis, I show that wheat has a *cor* (*co*ld-*r*egulated) gene, represented by cDNA clone pWG1, that is related to *Arabidopsis* cor*47*, a cold-regulated gene that encodes COR47, a "boiling-stable" polypeptide. I also present the DNA sequence of pWG1. The data indicate that the *cor* gene represented by pWG1 encodes a 39 kD hydrophilic polypeptide. The gene was designated *cor39* and the polypeptide COR39. The deduced amino acid sequence of COR39 indicates that it contains a lysine-rich sequence that is repeated six times. This same sequence is present in COR47 and Group II LEA proteins. In addition, COR39 has six glycine-rich repeats which are related to the repeats found in the barely and maize "dehydrins." Like *lea* transcripts, *cor39* transcripts accumulate in response to exogenous application of ABA and drought stress. The similarities and differences between *cor*39, *Arabidopsis cor* genes, and *lea* genes are discussed in terms of regulation of expression and possible roles in freezing and drought tolerance.

This dissertation is dedicated

to my husband,

Jiasheng Zhou

with love

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Chapter 1

Introduction

Plants are commonly subjected to a large number of environmental and biological stresses. These adverse conditions may interfere with normal growth and development of plants and can result in low quality and yield of crop plants. Low temperature is the single most limiting factor to natural plant distribution, and freezing stress is a major cause of crop loss (Burke *et al.*, 1976). Improving freezing tolerance of crops such as wheat is an important aspect of crop improvement. Understanding the principles of freezing injury and the mechanisms of freezing tolerance are important steps towards improving freezing tolerance of crops.

Plant Cold Acclimation

Some plant species increase in freezing tolerance during exposure to a period of low, nonfreezing temperature (Mazur, 1969; Burke *et al.*, 1976; Levitt, 1980 a). This process is termed cold acclimation. Some nonacclimated woody species are injured by temperature of about -10° C, but after cold acclimation, they can survive at -196° C (Weiser, 1970). Wheat and other winter cereals can also cold acclimate; nonacclimated plants are killed at about -5° C, while cold acclimated plants can survive to below -15 °C (Burke *et al.*, 1976). The freezing tolerance gained by plants during cold acclimation depends on the acclimation conditions, species and varieties of crops.

Freezing Process and Injury

The nature of the freezing process and the injury that it causes has been the subject of study for more than 100 years. There are many different reports and hypotheses (Steponkus, 1984). Briefly, the freezing process involves redistribution of water. When plant cells freeze, ice forms either intracellular or extracellular depending on the type of tissue or the cooling rate. In nature, extracellular ice formation occurs more often than

1976; Levitt, 1980 a; Steponkus, 1984). Extracellular ice formation occurs in the vicinity of the cell wall. The ice crystals in the extracellular free space are a water reservoir due to their low vapor pressure. As the temperature drops, the vapor pressure of the extracellular space is lower than that of the intracellular space. When the temperature drops slowly, plant cells achieve equilibrium either by cell dehydration or intracellular ice formation. In either case, the plasma membrane plays an important role in determining the manner of equilibration. If the plasma membrane is intact, the plasma membrane is an effective barrier to extracellular ice and the intracellular contents remain supercooled due to lack of effective ice nucleators (Steponkus, 1984). Because of the semipermeable characteristic of the plasma membrane, water moves to the outside space from inside the cell to achieve equilibrium. As water movement continues, the cell becomes dehydrated and injury can occur. There are many hypotheses on the mechanism of freezing injury caused by cellular dehydration including volumetric and area contraction, high salt concentration, pH change, eutectic crystallization, removal of water of hydration of macromolecules, oxidation of sulfhydryl groups and others (Mazur, 1969; Burke *et al.*, 1976; Levitt, 1980 a; Williams, 1981; Steponkus, 1984). However, few of these hypotheses are supported by direct evidences (Steponkus, 1984).

Ice adhesion can also cause freezing injury (Olien, 1974; Olien and Smith, 1981; Olien and Lester, 1985). Adhesion stress develops when the interfaces of growing ice crystals and hydrophilic substances of the cell wall or plasma membrane compete for interfacial liquid water. This causes adhesion between ice and the cell wall or plasma membranes. This kind of injury happens when the temperature is below -10° C.

Plants that lack the capacity to cold acclimate freeze intracellularly, as do plants which are able to cold acclimate, but are nonacclimated. Intracellular ice formation causes injury. The hypothesis for the mechanism of freezing injury caused by intracellular ice formation is mechanical rupture of the cell and deleterious physical contact of the membrane by ice (Mazur, 1969; Burke *et al.*, 1976).

From direct cryomicroscopic observations of isolated rye protoplasts (*Secale cereale L.* cv. Puma), Steponkus (1981) concludes that the primary site of freezing injury is the plasma membrane. He summarizes four kinds of injury: (a) Expansion-Induced Lysis. This occurs during warming and thawing, when water moves back into the protoplasts resulting in expansion and lysis of the protoplasts. Injury results from an irreversible loss of membrane material during plasmolysis. (b) Loss of Osmotic Responsiveness. Following cooling, the plasma membrane losses its semipermeable characteristics. This is probably due to the alterations in the bilayer structure of the plasma membrane and the loss of membrane proteins. (c) Altered Osmotic Behavior During Warming. Although the protoplasts are osmotically responsive, their volume after thawing is less than expected from the Boyle-van't Hoff relationship. This is probably due to either a prior transient loss of intracellular solutes or leakiness of the plasma membrane. (d) Intracellular Ice Formation Under Rapid Cooling Condition (3 ° C/min). Intracellular ice formation causes injury to the plasma membrane.

Mechanism of Cold Acclimation

A number of physiological, biochemical, and molecular changes have been shown to occur in plants during cold acclimation. Understanding the roles that these changes have in freezing tolerance and low temperature survival should ultimately lead to new methods to improve the freezing tolerance of important horticultural and crop plant species.

Physiological and Biochemical Changes

Photosynthesis and respiration are depressed by low temperature during cold acclimation. It has been noted that the more freezing tolerant varieties of winter wheat have much higher rates of photosynthesis than those of less freezing tolerant cultivars. The high photosynthetic rate of plants during cold acclimation provides energy sources for freezing tolerance (Barta and Hodges, 1970). In winter wheat, it is found that respiration occurs via glycolysis and the Krebs cycle during initial cold acclimation at 7 ° C (the 50% survival temperature after such cold acclimation is about -10°C), but shifts to the pentose phosphate pathway during the second stage of cold acclimation at 2° C (the 50% survival temperature after this stage of cold acclimation is about -17° C). This shift is believed to aid cold acclimated plants to maintain dormancy during brief warm winter weather (Olien and Smith, 1981).

In winter cereals, a large amount of fructan accumulates during cold acclimation (Olien and Lester, 1985). Fructan is the energy source for early spring growth. Rye, barely and wheat also undergo fructan conversion when cold acclimated seedlings are frozen for twenty four hours at -3° C. Fructans decrease while intercellular fructose and sucrose increase. The released fructose and sucrose are believed to have an important role in adhesion stress; they are in solution at equilibrium with ice so they can form an effect barrier to adhesion. Some arabinoxylan mucilages produced in the cell walls are believed to be effective inhibitors of ice crystal formation in extracellular spaces (Olien and Smith, 1981). The structure of the arabinoxylans from wheat and rye has been studied (Kindel *et al.*,1989). The rye arabinoxylans have different structures than the wheat arabinoxylans. These differences might count for the fact that the rye polysaccharide is a better inhibitor of ice formation than is that of wheat.

In winter wheat at 2° C, starch-sugar metabolism shifts; starch disappears and sugars accumulate (Olien and Smith, 1981). Other organic solutes and proline also accumulate during cold acclimation. The accumulation of small molecules is believed to play an important role in cyroprotection and freezing point depression (Levitt, 1980 a). However, very high concentrations of these small molecules are needed in order for them to have cyroprotective functions. In plants, it appears that these small molecules play cryoprotective functions mainly on a colligative basis (Volger and Heber, 1975).

Soluble proteins also accumulate during cold acclimation (Levitt, 1980 a). In spinach and cabbage, some of these soluble proteins have a high content of hydrophilic amino acids and are heat-stable (Volger and Heber, 1975; Hincha, *et al.*, 1989, 1990). *In vitro* cryoassay experiments indicate that these hydrophilic heat-stable soluble proteins, with the same concentration (ug/ml), these polypeptides were found to be greater than 15 times more effective in protecting thylakoid membranes against mechanical freeze-thaw damage *than control* protein such as BSA (bovine serum albumin). The concentration of these cryoprotective proteins in the leaves of cold acclimated plants appears to be high enough to contribute significantly to the freezing tolerance of plants. However, there is no direct evidence that these proteins have important cryoprotective roles *in planta*.

Many changes in lipid membrane composition occur during cold acclimation (Yoshida and Uemura, 1984; Lynch and Steponkus, 1987). Acclimated plants have significant increases in free sterols and phospholipids. The level of di-unsaturated species of phosphatidylcholine and phosphatidylethanolamine double in cold acclimated rye cells. There is direct evidence that these changes have dramatic effects on the cryobehavior of rye plant cells and that they contribute to the freezing tolerance of the plants (Steponkus, *et al.*, 1988). It also reported that the structure of chloroplast membranes is altered during cold acclimation (Vigh *et al.*, 1985).

Changes in Gene Expression During Cold Acclimation

It has been reported that cycloheximide, a protein synthesis inhibitor, can prevent cold acclimation in *Brassica napus* and wheat (Kacperska-Palacz *et al.*, 1977; Trunova, 1982). Thus, cold acclimation may require changes in gene expression. Many studies have been carried out in this area.

a. Isozyme Composition

Temperature effects the structure and function of enzymes. Temperature affects the hydrogen-bond and hydrophobic interactions that stabilize protein structure. Temperature can also affect the Km and activation energies for enzymes. Therefore, during cold acclimation, one might expect that certain enzymes would be modified, or that different forms might be synthesized. Indeed, it has been reported that acclimated and nonacclimated plants have different isozymes of ribulose bisphosphate carboxylase / oxygenase (Rubisco) (Huner and Macdowall, 1976 ab; 1979 ab). The "acclimated enzyme" has twice the specific activity of the nonacclimated enzyme. At 5° C, the acclimated enzyme has a lower Km and higher affinity for CO_2 than does the "nonacclimated isozyme." In contrast, at 25° C the nonacclimated enzyme has the lower Km. The acclimated enzyme is also more stable than the nonacclimated enzyme at -20° C. Some isozymes have different amino acid compositions indicating that they are encoded by cold-regulated genes (Shomer-Ilan and Waisel, 1975). It has been suggested that the acclimated enzymes might be better suited to low temperature and thus help plants survive better in lower temperature environments (Huner and Macdowall, 1979; Shomer-Ilan and Waisel,1975).

b. Protein Synthesis.

The soluble protein content of plants increases during cold acclimation. In wheat and black locust, it is found that soluble protein levels in acclimated plants are about 300% and 50%, respectively, greater than that in nonacclimated plants (Siminovitch *et al.*, 1968; Trunova, 1982). It has also been shown that changes in polypeptide composition occur during cold acclimation. These include increases and decreases in polypeptide levels, as well as novel changes. In alfalfa, cold treatment induces alterations in the membrane protein profile; about 10 new polypeptides are synthesized (Mohapatra *et al.*, 1987, 1989). In barley, cold shock induces some proteins and represses others (Cattivelli and Bartels, 1989). Interestingly, the changes that occur are different between winter and spring barley varieties. In spinach, exposure to 5° C induces the synthesis of three new proteins having molecular weights of 160, 117 and 85 kD (Guy and Haskell, 1987). These proteins are detected during the first day of cold acclimation and are synthesized for as long as the plants are kept at 5 °C (14 days was the longest time tested). During deacclimation (when plants are transferred back to the normal growth temperature), the synthesis of these three proteins markedly decreases. The induction of these proteins and the "depressions" during cold acclimation are highly correlated with the induction and loss of freezing tolerance. In *Arabidopsis*, newly synthesized polypeptides of 160, 47, 24, 15 and 6.6 kD occur during cold acclimation (Gilmour *et al.*,1988; Lin *et al.*, 1990; Gilmour *et al.*, *in press*), all of which share the unusual biochemical property of remaining soluble upon boiling.

High molecular weight proteins also accumulate during cold acclimation of wheat (Sarhan and Perras, 1987; Perras and Sarhan, 1989). It has been suggested that the most important protein is a 200 kD polypeptide. This polypeptide accumulates at higher concentration in a cold-tolerant cultivar (winter wheat) than in a cold-sensitive one (spring wheat). Six other polypeptides, 64, 52, 48, 47, 42 and 32 kD, have been shown to increase during cold acclimation. *In vivo* labeling experiments show that the 200 kD polypeptide is present in roots, crowns and leaves; the 36 kD polypeptide is present in leaves, and that the 52 and 64 kD polypeptides in roots; these proteins all expressed at a higher level in the freezing tolerant cultivar than that in the sensitive one.

All of these results suggest that cold-induced proteins might play important roles in freezing tolerance. Therefore, efforts are in progress to identify the genes encoding these proteins, to study the functions of these proteins in freezing tolerance and to study the regulation of their expression by low temperature.

c. mRNA Population Changes and Cold-regulated cDNA Clones

In vitro translation experiments have shown that changes in the concentrations of certain mRNAs occur during cold acclimation (Guy and Haskell, 1985, 1987; Mohapatra et al., 1987; Gilmour *et al.*, 1988). By constructing and screening cDNA libraries prepared from poly (A^+) RNA of cold-acclimated plants, cDNA clones of genes which are specifically expressed during cold-acclimation have been isolated. In alfalfa, three such clones have been identified (Mohapatra *et al.*, 1989). Northern analysis shows the accumulation of mRNAs corresponding to these cDNA clones is cold-acclimation specific; abscisic acid (ABA), drought and wounding stress do not significantly influence their accumulation. A positive correlation has been observed between the expression of these cloned sequences and the degree of freezing-tolerance in four alfalfa cultivars. In *Arabidopsis*, cDNA clones for four *cor* genes have been isolated (Hajela *et al.*, 1990). These genes are responsive to ABA and drought stress, but not to heat shock. Nuclear run-on transcription assays indicate that the low temperature regulated expression of three of the *cor* genes is controlled primarily at the posttranscriptional level, while the fourth is controlled at the transcriptional level. The functions of these cold-regulated genes and the regulation of their expression are being studied.

d. Effect of Abscisic Acid (ABA) on Cold Acclimation.

Endogenous levels of ABA have been shown to increase in some plants in response to low temperature (Dale and Campbell, 1981; Kacperska-Palacz, 1978; Chen and Li, 1982). Exogenous application of ABA at normal growth temperature can also improve freezing tolerance of certain plants. The freezing tolerance of cell suspension culture of winter wheat, winter rye and bromegrass increases in response to ABA (Chen and Gusta, 1983). The degree of cold hardiness and the rate of hardening obtained by ABA treatment is significantly higher than that caused by low temperature. It has been reported that exogenously applied ABA can also enhance the freezing tolerance of *Arabidopsis* (Lang *et al.*, 1989). The freezing tolerance of plants treated with ABA at a normal growth temperature (20° C) appeared to increase more rapidly than that of plants acclimated at low temperature (4° C). *In vivo* labelling experiments indicate that cold acclimation and ABA treatment can induce some of the same polypeptides. However, cold acclimation and ABA induced specific polypeptides as well.

The Relationship of Drought and Freezing Tolerance.

As mentioned above, plant cells become dehydrated during a freeze-thaw cycle. Therefore, freezing tolerance must include dehydration tolerance. Many studies have been carried out on the correlation of freezing tolerance with drought tolerance. Studies on red dog wood indicate that the freezing tolerance increases from -3 to -11° C after seven days of water-stressed treatment (Chen and Li, 1977). Water stress and short day treatment (woody species require short day to become cold acclimated) had a similar pattern of biochemical changes, specifically decreases in protein, RNAs and starch, and increases in soluble sugar. In winter wheat and rye, the same degree of freezing tolerance is acquired following a four-week cold acclimation and a twenty-four-hour desiccation stress(Cloutier,1983). These data suggest that there may be a common component in the mechanism of freezing and drought tolerance. Interestingly, all four cold-regulated genes that have been isolated from *Arabidopsis* are also induced by drought stress (Hejela *et al.*,1990)

Mechanism of Drought Tolerance

Plant responses to drought stress have been extensively studied. Similar to freezing stress, plants undergo numerous physiological and biochemical changes during acclimation to drought stress (Levitt, 1980 b). Osmotic adjustment is one of the major responses. Solutes such as proline, betaine, sucrose and fructans accumulate in plant cells in response to a decrease in osmotic potential. These changes help cells compete for water with the external physical environment. ABA also plays an important role in plant drought tolerance. Under drought stress, large amounts of ABA accumulate in leaves leading the closure of stomata to reduce the transpiration rate. At certain stages of plant development, ABA is also elevated. Embryo maturation, prior to seed desiccation, is one such stage (Dure *et al.*, 1989). As the ABA content increases, a set of proteins named LEA (late embryogenesis abundant) proteins are synthesized. These LEA proteins are probably universal in plant seeds. These proteins and their mRNAs accumulate in the embryo tissues of seeds as they approach maturity and begin to desiccate; they disappear when seeds are germinating. The *lea* mRNAs are not easily detected in leaves or roots of nonstressed plants, however, they can be detected in water stressed, or ABA-treated leaves or roots (Dure *et al.*, 1989). Many cDNA clones or genomic clones of these *lea* genes have been isolated, sequenced and characterized (Dure *et al.*, 1989; Mundy and Chua, 1988, Litts *et al.*, 1987). The data indicate that LEA proteins can divide into three groups based on sequence homology (Dure *et al.*, 1989).

Group II LEA proteins are the most interesting ones in terms of the relationship between drought and freezing tolerance, because the expression of RAB 21, a rice LEA Group II protein, is also regulated by low temperature and salt stress (Hahn and Walbot, 1989) and the deduced amino acid sequence of COR47 in *Arabidopsis,* a boiling-stable polypeptide, shares homology with Group II LEA proteins (Gilmour *et al., in press*). The Group II LEA proteins all contain two lysine-rich repeats and a serine repeat located a few amino acids upstream of the first lysine-rich repeat; the second lysine-rich repeat is always near the C-terminus of the protein. These LEA proteins contain neither cysteine nor tryptophan residues, but have high concentrations of glycine. The proteins are very hydrophilic and "boiling-stable;" i.e., the proteins remain soluble upon boiling (Close *et al.*, 1989; Dure *et al.*, 1989). These conserved features are believed to have important roles for the function of these proteins (Godoy, *et al.*, 1990).

Four cDNA clones of barley "dehydrins" and one cDNA a clone of a corn dehydrin have been isolated from water-stressed seedlings (Close *et al.*, 1990). The polypeptides deduced from the sequences of these cDNA clones indicate that the dehydrins share all the common structural features of Group II LEA proteins. The transcripts corresponding to each dehydrin cDNA clone are abundant in dehydrating, but not in well-watered seedlings. All of these dehydration-induced proteins are heat-stable.

Five ABA- and desiccation-responsive cDNA clones have been isolated from the resurrection plant (Pirkoqaki *et al.*, 1990). The sequences of two of cDNA clones indicated that the transcripts encode proteins related to Group II LEA proteins, The other cDNA clones represent gene that encode proteins that are unrelated to Group II LEA proteins. A tomato cDNA clone representing a *lea* gene has also been isolated (Godoy, *et al.*,1990). The expression of this gene is regulated by ABA and salt stress, but is not responsive to cold or wounding.

Rationale and Objectives

Cold acclimation is a complex process that involves a variety of biochemical and biophysical changes. The precise role that each of the changes has in cold acclimation, however, is not certain. Some may contribute directly to the freezing tolerance of acclimated cells. Others may contribute to the overall fitness of the plant for low temperature survival, which in turn, could indirectly affect freezing tolerance.

It is known that changes in gene expression occur during cold acclimation (Guy, 1990; Thomashow, 1990). Current research efforts are directed at identifying and isolating cold-regulated genes, determining the roles that these genes have in cold acclimation, and determining the mechanisms responsible for their cold-regulation. At present, very little is known about these genes. Do plants have related *cor* genes that are activated during the cold acclimation process? Is the regulation of expression of these genes similar among different plants? Are any of the polypeptides encoded by *cor* genes related at a structural or functional level. To begin to address these issues, I have chosen to compare *cor* gene structure and expression in wheat with that in *Arabidopsis*. Ultimately, studies on this area may lead to the development of new method to improve freezing tolerance in wheat. Improving the freezing tolerance of wheat could have significant effects on the yields and quality of this important world food source.

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Chapter 2

Comparison of Wheat and *Arabidopsis cor* Genes and Polypeptides

Summary

Previous studies have shown that changes in gene expression occur in wheat during cold acclimation. Here, I show that at least some of these changes are at the mRNA level. Further, some of the cold-induced mRNAs encode polypeptides which have the property of remaining soluble upon boiling, a property found in certain cold-induced polypeptides of *Arabidopsis*. cDNA cloning, and Southern and Northern analysis indicate that wheat has a *cor* (*co*ld-*r*egulated) gene that is related to *Arabidopsis cor47*, a cold-regulated gene that encodes a "boiling-stable" polypeptide of 47 kD. The importance of the cold-regulated boiling-stable polypeptides to cold acclimation and their possible role in freezing tolerance are discussed.

Introduction

In many plant species, exposure to low nonfreezing temperature results in enhanced freezing tolerance (Levitt, 1980). The process by which plants adjust to low temperature and increase freezing tolerance is termed cold acclimation. Wheat is a plant that can cold acclimate.

Cold acclimation is a complex process that involves biochemical and physical changes including the accumulation of sugars and soluble proteins, and alterations in membrane lipid composition (Levitt, 1980; Burke *et al.*, 1976; Lynch and Steponkus, 1987). However the roles that most of these changes have in cold acclimation and whether they contribute significantly to freezing tolerance *in planta* is uncertain.

It is known that changes in gene expression occur during cold acclimation (Guy, 1990; Thomashow, 1990). Research efforts are in progress to identify cold-regulated (*cor*) genes, to study their roles in cold acclimation and freezing, and to determine the mechanism of regulation that controls expression of these genes. Many fundamental questions need to be addressed. Do different plant species respond to low temperature in a similar way? Do different plants have related *cor* genes? Are *cor* genes products related at the structural and functional level? In this chapter I begin to answer some of these questions. I have found that although wheat is relatively unrelated to *Arabidopsis*, there are many similarities in the changes of gene expression that occur during cold acclimation in these two plants: wheat induces changes in mRNA populations; certain of the cold-induced wheat mRNAs, like *Arabidopsis cor* transcripts, encode polypeptides that have the unusual property of remaining soluble upon boiling; and wheat has at least one *cor* gene that is related to an *Arabidopsis cor* gene. A cDNA, pWG1, representing this wheat *cor* gene was isolated and its expression examined.

Materials and Methods

Plant Material

Winter wheat *Triticun aestivum L.* cv winoka were grown in controlled environment growth chambers. Temperature was maintained at 20° C (day and night) and the photoperiod was a 14/10 hour day/night cycle with fluorescent light. Light intensity was approximately 120u $Em^{-2}s^{-1}$. Plants were watered daily. Plants were grown under these conditions for two weeks, then were either harvested or transferred to a growth chamber at low temperature for various time periods for cold acclimation.

Freezing Test

Freezing tolerance of leaves was determined by the electrolyte leakage method of Sukumaran and Weiser (1972). Fifteen leaf discs randomly selected from plants of different pots were placed in stoppered culture tubes and incubated in a low temperature bath (Masterline Model 2095, Forma Scientific). The temperature in the bath was preset at -2° C. Ice chips were added to each tube to initiate freezing. After an overnight equilibration period, the temperature was lowered manually 1° C per hour. The samples were withdrawn at each temperature point, placed on ice and thawed overnight in a cold room (2° C). Three ml of distilled water was added to each tube and the tubes were shaken gently for 3 hours. The conductivity of each sample was measured using a conductance meter (YSI Model 35). For 100% leakage, each sample was frozen at -80° C without any solution for one hour, then the original solution was added back, the sample was shaken for another 3 hours, and the conductivity was measured. A plot of temperature versus percent electrolyte leakage was drawn. The LT_{50} (lethal temperature) was defined as the temperature that gave 50% electrolyte leakage.

In vitro Translation

Poly (A^+) RNA (1 ug) was translated *in vitro* in a 25 ul volume with rabbit reticulocyte lysate (Promega Biotec) using the procedure suggested by the manufacturer (Promega Technical Bulletin NO.3). The *in vitro* translation products, radiolabeled with ^{35}[S] methionine, were either directly separated by SDS-PAGE (Laemmli, 1970) or on two-dimensional polyacrylamide gels (2D-gels) (O'Farrell, 1975). For 2D-gels, the first dimension was an equilibrium IEF gel with a pH range from approximately 4 to 8 and the second dimension was a 10% SDS-PAGE gel. The gels were either dried directly or soaked in Amplify (Amersharm) for 15 minutes prior to drying. Kodak X-Omat AR5 X-ray film was used for both fluorography (exposed at -80° C) and autoradiography (exposed at room temperature).

Boiling Treatment of *in vitro* Translation Products

The *in vitro* translation products were diluted with 5 volumes of 50 mM Tris-HCl (pH7.5). The samples were boiled in a water bath for 10 minutes, then were centrifuged in an Eppendorf microfuge two times for 15 minutes to remove the insoluble material. Polypeptides that remained soluble were precipitated with 7 volumes of acetone and pelleted in microfuge for 15 minutes. The pellet was suspended in loading buffer [10% (v/v) glycerol, 0.01% (w/v) bromphenol blue, 2% (w/v) SDS, 60 mM Tris-HCl (pH 6.9), 100 mM dithiothreitol] and fractionated either by SDS-PAGE or 2D-gels as mentioned above.

RNA Extraction

Excised leaves were frozen in liquid nitrogen, pulverized using a mortar and pestle, and stored at -80° C prior to extraction. Total RNA was isolated using a modified version of method of Galau (1981). Frozen pulverized plant material was extracted in a buffer containing 100 mM Tris-HCl (pH 7.5), 100 mM NaCl, 50 mM EGTA, 1% (w/v) SDS, 10mM DTT, 6% (w/v) p-aminosalicylic acid (sodium salt), and 1% (w/v) tri-isopropylnaphthalenesulfonic acid (sodium salt), then extracted with an equal volume of buffer-saturated PCI [phenol: chloroform: isoamyl alcohol, 25: 24: 1 (v/v/v)]. After centrifugation, the aqueous (top) phase was extract once more with PCI, followed by ethanol precipitation. The pellet was dissolved in water, then precipitated twice on ice with 2 M LiCl to purify RNA from DNA. The RNA was finally precipitated with ethanol, dissolved in distilled water, and stored at -80° C.

Poly (A^+) RNA was obtained using a poly (U) sepharose column (Sigma) based on the method of Cashmore (1982). RNA was dissolved in an equal volume of 2X S buffer [1X S buffer is 0.5 M NaCl, 10 mM Tris-HCl (pH8.0), 1mM EDTA, 0.1% (w/v) SDS], and heated for 10 minutes at 65° C. The solution was to cooled on ice and applied to a poly (U) sepharose column which was previously washed with 90% formamide followed by 1X S buffer. The flow-through was reheated and reapplied to the column. The column was washed with TE [10 mM Tris-HCl (pH 8.0), 1mM EDTA] and SDS [0.1% (w/v)] and the poly (A^+) RNA eluted with 90% formamide. The formamide fraction was ethanol precipitated, and the pellet was dissolved in distilled water and stored at -80° C.

DNA Extraction

Large scale preparations of plasmid DNA were extracted from *E. coli* by alkaline lysis and banded on isopycnic CsCl-ethidium bromide gradients according to standard method of Maniatis (1982). Mini-preparations of plasmid DNA were extracted from *E. coli* by the boiling method (Holmes and Quigley, 1981). Overnight cultured cells (1.5 ml) were pelleted and suspended in STEP buffer [8% (w/v) sucrose, 5% (v/v) triton X100, 50 mM EDTA, 50 mM Tris-HCl (pH 8)], boiled for 50 seconds and then centrifuged in an eppendorf microfuge for 10 minutes. The plasmid DNA in the top supernatant was precipitated by adding an equal amount of isopropanol and centrifuging in a microfuge. Then DNA pellet was dried and dissolved in TE buffer and stored at -20° C

Construction and Screening of a cDNA Library

Poly (A^+) RNA isolated from the leaves of 3 week cold acclimated (2° C) plants (Winoka) was used to synthesize double-strand cDNA according to Gubler and Hoffman (9183). The doubled-stranded cDNA was blunt-ended, and methylated with *E. coli* methylase. *Eco*RI linkers were ligated to the cDNAs and the fragments were cut with *Eco*RI, purified and ligated to the *Eco*RI site of Lambda ZAP (Stratagene). There were approximately 10^5 recombinants in the cDNA library. The library was amplified once and stored in either SM buffer [100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl (pH 7.5), 0.01% (w/v) gelatin] containing 0.05% chloroform at 4° C or in SM buffer with 7% (w/v) DMSO frozen at -80° C.

For screening the library, plague lifts were made using Nitran membranes (Schleicher and Scheull) according to standard methods (Maniatis *et al.*,1982). The filters were baked at 80° C for one hour in a vacuum oven. Before hybridization, the filters were first washed with 0.1X SSC (1X SSC is 0.15 M NaCl, 0.015M sodium citrate), 0.5% (w/v) SDS at 60° C for 30 minutes. Plaques were probed with labeled cDNA inserts from pHH7.2, pHH28, pHH29 and pLCT10, which are all cold-induced *Arabidopsis* cDNA clones (Hajela *et al.*, 1990). The inserts were labeled with [^{32}P] CTP by the random priming method (Fienberg *et al.*, 1983). Hybridization conditions were 6X SSC, 0.5% (w/v) SDS, 0.25% (w/v) nonfat dry milk at 60° C overnight (Johnson *et al.*, 1984). The filters were washed three times with 2X SSC, 0.5% (w/v) SDS at room temperature, each time for 5 minutes, then washed two times with 2X SSC, 0.5% (w/v) SDS at 60° C for 30 minutes. Plagues showing homology were further purified and the recombinant cDNAs were excised from the phage in pBluescript SK^- by biological rescue using the method provided by the manufacturer (Stratagene).

Northern and Southern Analysis

Total or poly (A^+) RNA was fractionated on denaturing formaldehyde agarose gels using standard methods (Maniatis, 1982). One ul of ethidium bromide (400 ug/ml) was added to the sample buffer before the sample was denatured at 65° C for 15 minutes (Rosen *et al.*, 1990). This allowed visualization of RNA in the gel using UV light. The RNA was transferred to Nytran membranes (Schleicher and Schuell) with 10X SSPE buffer [1X SSPE is 18 mM NaCl, 10 mM NaH_2PO_4 (pH 7.7), 1 mM EDTA]. The blots were stained with methylene blue [0.02% (w/v) methylene blue in 300 mM of NaOAC (pH 5.5)] to check the efficiency of transfer. The blots were baked at 80° C in a vacuum oven for 1 hour.

Plasmid DNA was digested with restriction enzymes and fractionated on 1% agarose gels using standard methods (Maniatis, 1982). DNA gels were denatured with 0.5 M NaOH in 1.5 M NaCl for 1 hour, and neutralized with 0.5 M Tris-HCl (pH 8.0) in 1.5 M NaCl. The DNA was transferred to Nytran membranes using the same method as with RNA gels. The blots were baked as described for the Northern blots.

Northern and Southern blots were prewashed with 0.1X SSPE, 0.5% (w/v) SDS at 60° C for 30 minutes. Prehybridization and hybridization of the Southerns were in 6X SSPE, 0.5% (w/v) SDS, 0.25% (w/v) nonfat milk at 60° C. The wash was in 0.1X SSPE, 0.5% (w/v) SDS at 65° C. Northern blots were hybridized in 5X SSPE, 50% (v/v) formamide 42° C, and washed in 0.1X SSPE, O.5% (w/v) SDS, 55° C using standard methods (Maniatis, 1982). Gel purified cDNA inserts were radiolabeled with ^{32}[P] CTP by the random priming procedure (Fienberg *et al.*, 1983). Kodak AR5 X-ray film and intesifying screens (Dupont) were used for autoradiography. Films were exposed at -80° C for various times depending on the amount of radioactivity

Results

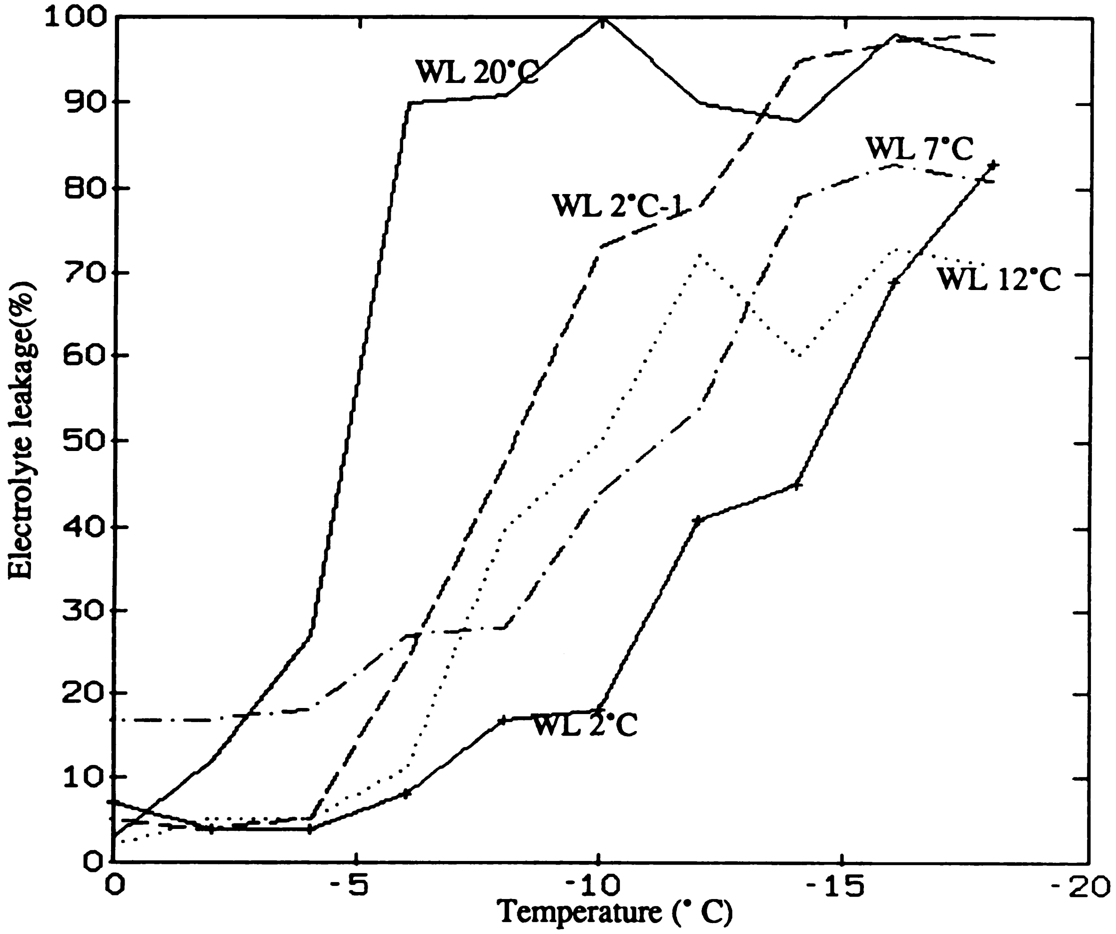
Freezing Tolerance of Cold Acclimated Plants

The freezing tolerance of winter wheat Winoka increased upon treatment at low temperature. The degree of freezing tolerance enhancement depended on the cold acclimation conditions: the lower the temperature above 0° C and the longer the period of cold acclimation, the greater the freezing tolerance (Figure 1). Specifically, the LT_{50} of nonacclimated plants was -5° C, while the LT_{50} of plants that were acclimated at 2° C for three weeks was -14° C. Plants which were acclimated at 2° C for only one week had an LT_{50} of -8 ° C. The LT_{50} of plants acclimated at 12° C for three weeks was -10° C, while the LT_{50} of plants acclimated at 7° C was -11° C.

Changes in Gene Expression During Cold Acclimation

In vitro translation of poly (A^+) RNA isolated from cold acclimated and nonacclimated plants showed that the translatable mRNA populations were different in the cold acclimated and nonacclimated plants (Figure 2). Specifically, there were a marked increases in the levels of ten polypeptides in the *in vitro* translated RNA from cold acclimated plants. The largest among these ten polypeptides had a molecular weight 200 kD and pI of approximately 6.7. Another polypeptide with similar pI (about 6.8) was smaller, 180 kD. There were five polypeptides, 80, 48, 47, 18, and 17 kD, with slightly basic pIs ranging from 7.4-7.7. Three other acidic polypeptides had molecular weights

Figure 1. Effect of low temperature and period of cold acclimation on freezing tolerance of winter wheat *T. aestivum* cv Winoka. WL20: non acclimated plants. WL12: plants were acclimated at 12°C for three weeks. WL7: plants were acclimated at 7 ° C for three weeks. WL2: plants were acclimated at 2° C for three weeks. WL2-1: plants were acclimated at 2° C for one week.



Electrolyte leakage(%)
100
90
80
70
60
50
40
30
20
10
0
0
-5
-10
-15
-20
Temperature (° C)
WL 20°C
WL 2°C-1
WL 7°C
WL 12°C
WL 2°C

Figure 1

Figure 2. Two-dimensional electrophoretic analysis of *in vitro* translation products of poly (A^+) RNA isolated form nonacclimated (A) or acclimated (B) (2° C three weeks) *T. acestivum* cv Winoka. Circles indicate translation products that increased in cold acclimation plants.

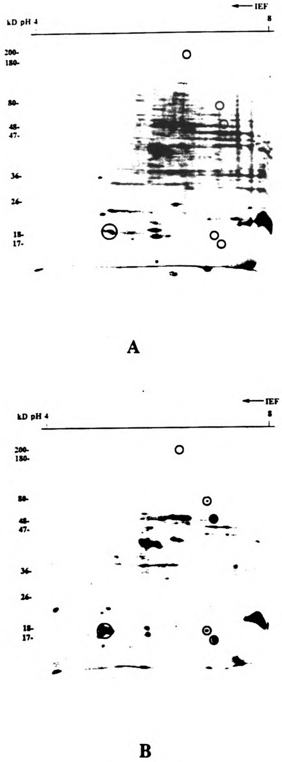

IEF
kD pH 4
8
200-
180-
80-
48-
47-
36-
26-
18-
17-
A
IEF
kD pH 4
8
200-
180-
80-
48-
47-
36-
26-
18-
17-
B

Figure 2

around 18 kD with pIs of 4.7-4.9. The 48 and 47 kD polypeptides were the most strongly induced of the ten cold-regulated polypeptides. In Figure 2, in order to show the strong induction of these cold-induced polypeptides, the autoradiography of B is less exposure than A. These changes of *in vitro* translation products were consistent, being observed in at least four independent experiments in which the plants were cold treated at three different temperatures, 12°C, 7° C and 2° C (data not shown). Similar changes were observed in another winter wheat variety, Genesee (data not shown).

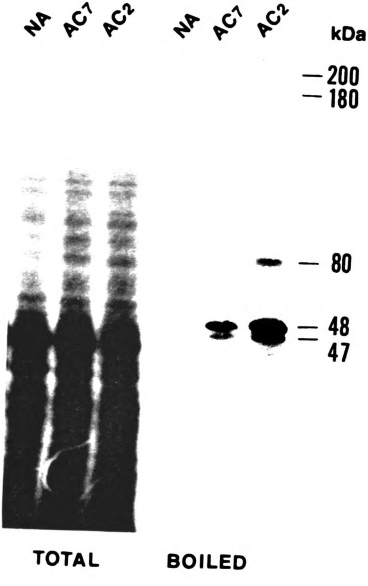
Cold Regulated mRNAs Encoding "Boiling-Stable" Polypeptides

Most proteins are denatured upon boiling, coagulate, and can be pelleted by centrifugation. However, it has been shown in *Arabidopsis* that mRNAs encoding "boiling-stable" polypeptides accumulate during cold acclimation. This cold acclimation response also occurs in wheat. Figure 3 shows the results of *in vitro* translation products of transcripts isolated from control acclimation plants fractionated either directly by SDS-PAGE (Total) or after boiling and centrifugation to remove insoluble material (Boiled). The data indicates that most of proteins were removed by boiling. However, the 200, 180, 80, 48, and 47 kD polypeptides translated from cold acclimated mRNA remained soluble. The other five polypeptides with molecular weights of around 18 and 17 kD were also boiling stable, but cannot be seen in Figure 3 because they ran off the gel. The results of 2D-gels showed that all of the cold-regulated boiling-stable, polypeptides had the same molecular weights and pIs as the cold-regulated polypeptides described in Figure 2 (data not shown).

Isolation of a Wheat Gene Related to *Arabidopsis cor47*

Transcripts encoding boiling-stable polypeptides of 160, 47, 24, 15, and 6.6 kD accumulate in cold acclimated *Arabidopsis* (Lin *et al.*, 1990; Gilmour and Thomashow, unpublished results). cDNA clones corresponding to four of these COR polypeptides were isolated (Hejela, *et al.*, 1990; Lin *et al.*, 1990; Gilmour and Thomashow, unpublished results); cDNAs pHH28, pHH7.2, pLCT10 and pHH29 correspond to the 160, 47,15, and 6.6 kD polypeptides, respectively. It was of interest to know if wheat had *cor* genes related to any of these *Arabidopsis cor* genes. Therefore, a cDNA library was constructed using poly (A^+) RNA isolated from cold acclimated (2° C, 3 weeks) Winoka and the library was screened with the four different cold-induced cDNA clones from *Arabidopsis*. Wheat

Figure 3. SDS-PAGE analysis of *in vitro* translation products of poly (A^+) RNA isolated from cold acclimated and nonacclimated plants. Total: *in vitro* translation products directly separated on SDS-PAGE. Boiled: boiling-stable *in vitro* translation products separated on SDS-PAGE. NA: nonacclimated. AC7: acclimated at 7° C for 3 weeks. AC2: acclimated at 2°C for 3 weeks.



b

Figure 3

Figure 4. Northern analysis indicating the accumulation transcripts hybridizing with pWG1 in cold acclimated wheat. A: RNA was isolated from cold acclimated plants(2° C, 3 weeks). B: RNA was isolated for nonacclimated plants. 15 ug of total RNA was fractionated on a formaldehyde agarose gel, transferred to a Nytran membrane and hybridized with ^{32}P-labeled pWG1 insert.

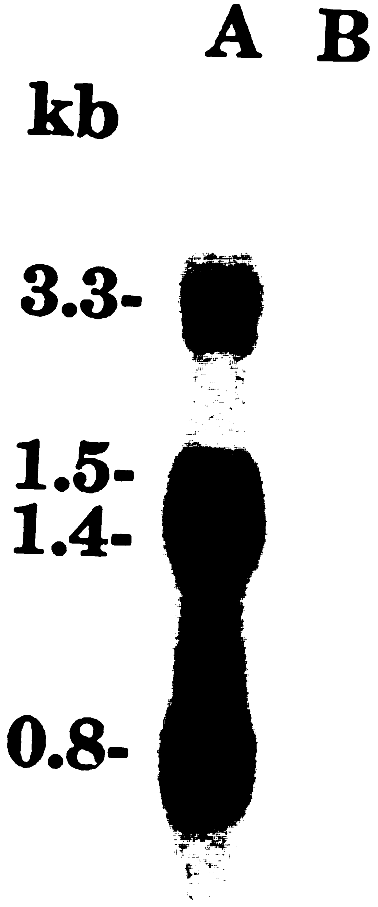


Figure 4

Figure 5. Southern analysis indicating the homology between *Arabidopsis cor47* and the wheat gene represent by pWG1. (a) Restriction fragments of pHH7.2 were fractionated on an agarose gel. digests were: E, *EcoR*I; B, *EcoR*I plus *BamH*I; K, *EcoR*I plus *Kpn*I; X, *EcoR*I plus *Xba*I.
(b) Southern blot of gel in (a) hybridized with pWG1 cDNA insert.
(c) Restriction map of the pHH7.2 cDNA insert. abbreviation are: E, *EcoR*I; X, X*ba*I; K, *Kpn*I; B, *BamH*I.

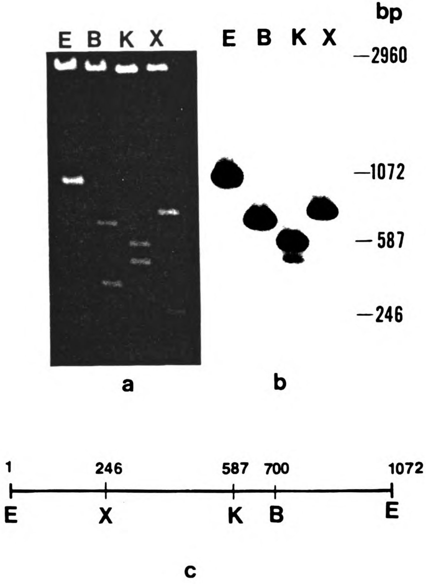


Figure 5

clones that hybridized with pHH 7.2 were detected. These clones were purified and further analyzed.

Northern analysis indicated that one of the clones that hybridized with pHH7.2, designated pWG1, represented a cold-regulated (*cor*) gene (Figure 4). Transcripts homologous to pWG1 were only present in cold-acclimated plants. The sizes of the transcripts were 3.3, 1.5, 1.4, and 0.8 kb.

Southern analysis indicated that the DNA sequence homology shared by pWG1 and pHH7.2 mapped to the middle region of pHH7.2 (between the *Xba*I and *Kpn*I restriction sites, Figure 5). DNA sequence analysis indicates that this region of pHH7.2 is within the coding sequence of the COR47 polypeptide (Gilmour *et al., in press*).

The wheat cDNA library was also screened for clones related to pHH29, pHH28 and pLCT10. No hybridization was detected with pLCT10. For pHH29, some clones showed hybridization and were further purified and studied. However, it was found that the homology was only to the 3' untranslated region of pHH29. The homology was probably due to the poly A tail (data not shown). For pHH28, all clones showed a similar low degree of hybridization. When the 3' end of pHH28 was digested with restriction enzymes to get rid of the poly A tail, and the wash conditions for the hybridization were changed to higher stringency [0.1X SSC, 0.5% (w/v) SDS at 65° C], similar results were obtained (data not shown). The significance of this hybridization remains unknown.

Discussion

In vitro translation experiments indicate that cold acclimation in wheat is associated with increased levels of transcripts encoding polypeptides of 200, 180, 80, 48, 47, 18, and 17 kD. The 200 and 180 kD polypeptides have similar pIs (about 6.8) as do the 48 and 47 kD polypeptides (about 7.5). Perras and Sarhan (1989) reported *in vivo* labeling experiments indicating a 200 kD polypeptide with pI 6.8 is induced during cold acclimation. It seems probable that this polypeptide is the same as the 200 kD polypeptide I found in my *in vitro* translation experiments. If this is true, then this polypeptide is not being processed *in vivo*.

The data presented indicate that many of the wheat polypeptides encoded by cold-regulated mRNAs share the unusual property of remaining soluble upon boiling. Similar results have been obtained with *Arabidopsis* (Lin *et al.*, 1990). Given the evolutionary distance between wheat and *Arabidopsis*, it seems probable that the accumulation of boiling-stable polypeptides will be found to be a common response of plants, at least among those that cold acclimate. In addition, I found that wheat has at least one gene that is related to an *Arabidopsis cor* gene that encodes a boiling-stable polypeptide, specifically *cor47*, and that this wheat gene is cold-regulated. These results suggest that the boiling stable-polypeptides probably have an important role in cold acclimation; two distantly related plants would not be expected to express genes that encode polypeptides with unusual biochemical properties unless they have some important functions. What functions might the boiling-stable COR polypeptides have? One possibility is that they might have cryoprotective properties. It has been reported that leaf cryoprotective polypeptides are synthesized in spinach and cabbage during cold acclimation (Hincha, *et al.*, 1990). With the same concentration (ug/ml), these polypeptides were found to be greater than 15 times more effective in protecting thylakoid membranes against mechanical freeze-thaw damage *(in vitro)* than control protein like BSA. These polypeptides, like the boiling-stable *Arabidopsis* and wheat COR polypeptides, are cold-regulated and remain soluble upon boiling. Interestingly, preliminary *in vitro* cryoassays have been carried out with COR 15 (the 15 kD boiling-stable polypeptide encoded by *Arabidopsis cor15*) that indicate that it has potent effect in protecting lactate dehydrogenase against freeze inactivation (Lin and Thomashow, unpublished data). However, these experiment were performed *in vitro*, whether these cold-induced boiling-stable polypeptides have cryoprotective function *in vivo* remains unknown.

I used four *Arabidopsis* cDNA clones, corresponding to *cor* encoding boiling-stable polypeptides of 160, 47, 15 and 6.6 kD, to screen the wheat cDNA library for related genes. Only *cor47* showed specific homology to a wheat gene. Immunoprecipitation experiments indicated that an antibody against COR160 kD (Gilmour, unpublished data) did not recognize any wheat polypeptides (data not shown). Thus, wheat does not appear to have genes structural related to *Arabidopsis* genes *cor160, cor15*, and *cor6.6*. However, Additional studies are needed to prove this because there is a possibility that the size of Winoka cDNA library (10^5 recombinants) is not big enough, therefore, the level of clones related to *Arabidopsis cor* genes is too low to detect.

Northern analysis indicated that there are many different sizes of cold-regulated mRNAs that hybridize with pWG1. Do these different transcripts originate from the same gene, representing different sites of transcript initiation or different processing events? Alternatively, are they transcribed from related genes or different members of a gene family? Additional experiment will be required to distinguish between these possibilities.

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Chapter 3

Molecular Characterization of pWG1, a cDNA Representing a Cold-Regulated Gene

Summary

In the previous chapter, I showed that similar to *Arabidopsis,* certain wheat *cor* (cold-regulated) genes encode boiling-stable polypeptides. A cDNA clone, pWG1, for one of these *cor* genes was isolated. This gene is related to *cor47* of *Arabidopsis* gene. In this chapter I present the DNA sequence of pWG1. The data indicate the *cor* gene represented by pWG1 encodes a 39 kD hydrophilic polypeptide. The gene was designated *cor39* and the polypeptide it encodes COR39. The deduced amino acid sequence of COR39 indicates that it contains a lysine-rich sequence that is repeated six times. Nearly identical lysine repeats are present in COR47 and Group II LEA (late embryogenesis abundant) proteins. In addition, COR39 has six glycine-rich repeats which are also related to repeats found in barely and maize "dehydrins" proteins that are members of the Group II LEA proteins family. Like *lea* transcripts, *cor*39 transcripts accumulate in response to ABA treatment and drought stress. The similarities and differences between *cor39*, A*rabidopsis cor* genes and LEA proteins are discussed in terms of regulation of expression and possible roles in freezing and drought tolerance.

Introduction

Changes in gene expression occur during cold acclimation (Guy, 1990; Thomashow, 1990). A major task now is to determine whether these cold-regulated genes play an important role in freezing tolerance. A number of cold-regulated genes have been isolated and are being characterized. In alfalfa, it has been shown that the levels of expression of three cold-regulated genes correlate positively with the freezing tolerances of four different cultivars (Mohapatra *et al.*, 1989). In *Arabidopsis*, cDNA clones of four cold-regulated (*cor*) genes have been isolated and being characterized (Hajela *et al* 1990). The transcript levels of these four *cor* genes increase markedly upon cold treatment (between 1 to 4 hours), remain at elevated levels for as long as the plants are kept at low temperature, and decrease rapidly to normal levels when the plants are deacclimated (transferred back to normal growth temperature). The expression of these *cor* genes is also regulated by ABA and drought stress. DNA sequence analysis indicates that these four *cor* genes encode hydrophilic polypeptides (Gilmour *et al.*, 1991; Lin and Thomashow, in preparation; Thomashow *et al.*, in preparation). Interestingly, the deduced amino acid sequence of COR47, a boiling-stable polypeptide, shares homology with Group II LEA (late embryogenesis abundant) proteins (Gilmour *et al.*, *in press*). LEA proteins have been hypothesized to have roles in drought tolerance (Dure *et al.*, 1989). These proteins are synthesized just prior to seed desiccation and are induced in water-stressed tissue or tissue that has been treated with ABA. LEA proteins are also hydrophilic and boiling-stable. Given the fact that freezing injury results in part from dehydration, plant freezing tolerance should include dehydration tolerance. The result that COR47 sequence shares homology with LEA proteins suggests that freezing and drought tolerance may involve related genetic mechanisms.

In the previous chapter I showed that similar to *Arabidopsis*, certain *cor* genes of wheat encode polypeptides that are boiling-stable. I also showed that the wheat *cor* gene represented by the cDNA clone pWG1 is related to the *cor47* gene of *Arabidopsis*. In this chapter I report the DNA sequence of pWG1. The data indicate that this cDNA represents a wheat *cor* gene, designated *cor39*, that encodes a hydrophilic, boiling-stable polypeptide of 39 kD. Further, the data indicate that COR39 is related to Group II LEA proteins. Gene expression studies indicated that *cor39* is regulated similarly to *Arabidopsis cor* genes. The possible roles of these *cor* genes in freezing and drought tolerance are discussed.

Materials and Methods

Plant Materials

Winter wheat *Trticum aestivum L.* cv Winoka grown in a controlled environment growth chamber for two weeks as described in Chapter II. The plants were then either harvested or given different treatments:

<u>Temperature Treatment.</u> Plants were placed in the growth chambers preset to various

desired temperatures for different time periods.

Drought Stress Treatment. Plants were placed in growth chambers under normal growth conditions. Drought stress was induced by withholding water until plants became visibly wilted (1-2 weeks).

ABA Treatment. Plants were sprayed to runoff with 100 um ABA (mixed isomers, Sigma) in 0.02% (v/v) polyethylene sorbitan monolaurate 20 (Tween-20). The pots were covered with Saran Wrap to slow evaporation and placed in a chamber under normal growth conditions for various times. Control plants were sprayed with a solution of 0.02% (v/v) Tween-20.

RNA and DNA Extraction

RNA was extracted from leave tissue and plasmid DNA was prepared as described in Chapter II. Genomic DNA from wheat was prepared from the supernatant left after LiCl precipitation of RNA during RNA extraction (Hejela *et al.*, 1990). The supernatant was diluted to 0.5 M LiCl with distilled H_2O and the DNA was precipitated with ethanol. The pellet was resuspended in TE [10 mM Tris-HCl (pH 8.0), 1 mM EDTA]. The resulting solution was extracted twice with PCI (as described in Chapter II Material and Methods), then precipitated with ethanol. The DNA was dissolved in TE and stored at 4° C.

Northern and Southern Analysis

Northern analysis was carried out as described in Chapter II. For Southern analysis, genomic DNA from wheat (about 20 ug) was digested with various restriction enzymes and fractionated on an agarose gel. Blots were prepared and hybridized as described in Chapter II except that the gel was treated with 0.25 M HCl for 15 minutes before it was denatured in alkaline solution.

Construction of pWG2

pWG1 was digested with *Eco*RI, the mixture ligated with T_4 DNA ligase and the preparation transformed into *E. coli*. A clone having the pWG1 insert in reverse orientation was isolated and designated pWG2.

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In vitro Transcription /Translation Experiments

pWG1 and pWG2 were linearized by digestion with *Hind*III and the inserts were transcribed *in vitro* with T_3 RNA polymerase (Stratagene) at room temperature for 2 hours using the T_3 promoter on the pBluescript KS$^-$ vector. The *in vitro* transcription products were treated with DNase I at 37 °C for 15 minutes and then extracted with PCI. The solution was precipitated with ethanol, the pellet resuspended in TE and translated *in vitro* as described in Chapter II.

DNA Sequence Analysis

The DNA sequence of the cDNA insert in pWG1 was determined on both strands. 5' and 3'deletions were generated by digestion with exonuclease III and mung been nuclease based on the method of Henikoff (1987). DNA sequencing was carried out on either single or double stranded DNA templates by the dideoxy chain termination reaction using sequenase (US Biochemical Corp) according to the supplier. Premature termination, which was a significant problem, was minimized by performing the termination reaction at 50° C and using Tagtrack sequencing system (Pomega). Single stranded plasmid DNA was prepared from *E. coli* strain MV1190 using the helper phage M13K07 (Vieira and Messing 1987). Double stranded DNA for sequencing was prepared according to Zhang *et al.* (1988).

Programs of the University of Wisconsin Genetic Computer Group were used for nucleic acid and protein sequence analysis. HIBio DNAsis and HIBio Prosis programs from Hitachi Engineering Co. Ltd. were also used. Hydropathy plots were conducted using a window of 9 amino acid residues according to Kyte and Doolittle (1982). TFASTA (Pearson and Lipman 1988) and WORD SEARCH (Wilbur and Lipman 1983) program were used for amino acid and nucleotide sequence comparisons.

Results

DNA Sequence Analysis

The DNA sequence of pWG1 is presented in Figure 6. The cDNA insert contains one long open reading frame of 1174 nucleotides that would encode a polypeptide of 391 amino acids. The calculated molecular weight of this polypeptide is 39.1 kD and its isoelectric point (pI) is 7.48. *In vitro* transcription/translation of the pWG1 insert in the "correct" 5'>3'

Figure 6. Nucleotide and deduced amino acid sequences of pWG1. The single letter amino acid code is shown above the first nucleotide of each codon. Single underlining indicates the lysine-rich repeat (KR); double underlining indicates the glycine-rich repeats (GR); dash line indicates overlapping regions of repeats.

```
GTAAACACATCAGCACTAGTAGATTTCACGAGTCAGAAGCTCAGCGCAAGATGGAGAACC   60
                                                   M  E  N      3

AGGCACACATCGCCGGCGAGAAGAAGGGCATCATGGAGAAGATCAAGGAGAAGCTCCCCG  120
Q  A  H  I  A  G  E  K  K  G  I  M  E  K  I  K  E  K  L  P
                                             KR1
GCGGCCACGGCGACCACAAGGAGACCGCTGGTACCCACGGGCACGCCGCCACGGCGACGC  180
G  G  H  G  D  H  K  E  T  A  G  T  H  G  H  A  A  T  A  T     43

ATGGTGCCCCGGCCACCGGTGGTGCCTACGGGCAGCAGGGTCACGCTGGAACCACCGGCA  240
H  G  A  P  A  T  G  G  A  Y  G  Q  Q  G  H  A  G  T  T  G     63
                                 GR1
CGGGGTTGCATGGCGCCCACGCCGGCGAGAAGAAGGGCGTGATGGAGAACATCAAGGACA  300
T  G  L  H  G  A  H  A  G  E  K  K  G  V  M  E  N  I  K  D     83
                                       KR2
AGCTCCCTGGTGGCCACGAGGACCACCAGCAGACCGGTGGCCACTACGGGCAGCAGGGAC  360
K  L  P  G  G  H  E  D  H  Q  Q  T  G  G  H  Y  G  Q  Q  G    103

ACGCCGGCACGGCGACGCATGGCACCCCGGCTACCGCTGGCACCTATGGG]CAACAGGGGC 440
H  A  G  T  A  T  H  G  T  P  A  T  A  G  T  Y  G  Q  Q  G    123
               GR2                          --------
ATACCGGCACGGCGACGCATGGCACCCCAGCGACCGGTGGCACCTATGGGGAGCAGGGAC  520
H  T  G  T  A  T  H  G  T  P  A  T  G  G  T  Y  G  E  Q  G    143
                  GR3
ACACCGGAGTGACCGGCACGGGGACGCACGGCACCGGCGAGAAGAAGGGCCTCATGGAGA  580
H  T  G  V  T  G  T  G  T  H  G  T  G  E  K  K  G  L  M  E    163

ACATCAAGGAGAAGCTCCCTGGTGGCCATGGTGACCACCAGCAGACCGCTGGCACCTACG  640
N  I  K  E  K  L  P  G  G  H  G  D  H  Q  Q  T  A  G  T  Y    183
                KR3
GGCAGCAGGGACACGTCGGCACGGGGACACATGGCGCCCCGGCTACCGGCGGGGCCTACG  700
G  Q  Q  G  H  V  G  T  G  T  H  G  R  P  G  Y  R  R  G  Y    203
            GR4
GGCAGCATGAACACGCCGGAGTGGCCGGCGCGGGAACATACGGCACCGGCGAGAAGAAGG  780
G  Q  H  E  H  A  G  V  A  G  A  G  T  Y  G  T  G  E  K  K    223

GCGTCATGGAGAACATCAAGGACAAGCTCCCTGGCGGCCACGGCGACCACCAGCAGACCG  840
G  V  M  E  N  I  K  D  K  L  P  G  G  H  G  D  H  Q  Q  T    243
                                  KR4
GTGGCACCTACGGGCAGCAGGGACACACCGGCACGGCGACGCATGGCACCCCGGCCGGCG  900
G  G  T  Y  G  Q  Q  G  H  T  G  T  A  T  H  G  T  P  A  G    263
-----                                  GR5
GCGGCACCTATGAGCAGCACGGACACACCGGGATGACCGGCACGGGGACACACGGCACCG  960
G  G  T  Y  E  Q  H  G  H  T  G  M  T  G  T  G  T  H  G  T    283

GCGAGAAGAAGGGCGTCATGGAGAACATCAAGGAGAAGCTCCCCGGTGGCCACGGCGACC 1020
G  E  K  K  G  V  M  E  N  I  K  E  K  L  P  G  G  H  G  D    303
                            KR5
ACCAGCAGACCGGTGGAGCCTACGGGCAGCAGGGACACACCGGCACGGCGACGCATGGCA 1080
H  Q  Q  T  G  G  A  Y  G  Q  Q  G  H  T  G  T  A  T  H  G    323
              -----                              GR6
CTCCGGCTGGCGGCGGCACCTACGGGCAGCATGCACACACTGGAATGACCGGCACGGAGA 1140
T  P  A  G  G  G  T  Y  G  Q  H  A  H  T  G  M  T  G  T  E    343

CGCACGGCACCACGGCCACCGGCGGCACCCATGGGCAGCACGGACACGCCGGAACGACTG 1200
T  H  G  T  T  A  T  G  G  T  H  G  Q  H  G  H  A  G  T  T    363

GCACTGGGACACACGGCACCGACGGGGTGGGCGAGAAGAAGAGCCTCATGGACAAGATCA 1260
G  T  G  T  H  G  T  D  G  V  G  E  K  K  S  L  M  D  K  I    383
                                                   KR6
AGGACAAGCTGCCTGGACAGCACTGAGCCCGGTGTGCCGACGG                  1303
K  D  K  L  P  G  Q  H                                         391
```

Figure 6

Figure 7a. Two-dimensional electrophoretic analysis of boiled *in vitro* transcription/ translation products of pWG1 cDNA insert. A, boiled *in vitro* transcription/ translation products of pWG1 cDNA insert was separated on a 2-D gel; B:boiled *in vitro* translation products of poly (A^+) RNA isolated from cold-acclimated plant was fractionated on SDS-PAGE.

Figure 7b. Two-dimensional electrophoretic analysis of boiled *in vitro* translation products of poly (A^+) RNA isolated form cold acclimated plants. A, boiled products fractionated on a 2-D gel; B, boiled products ractionated directly on SDS-PAGE.

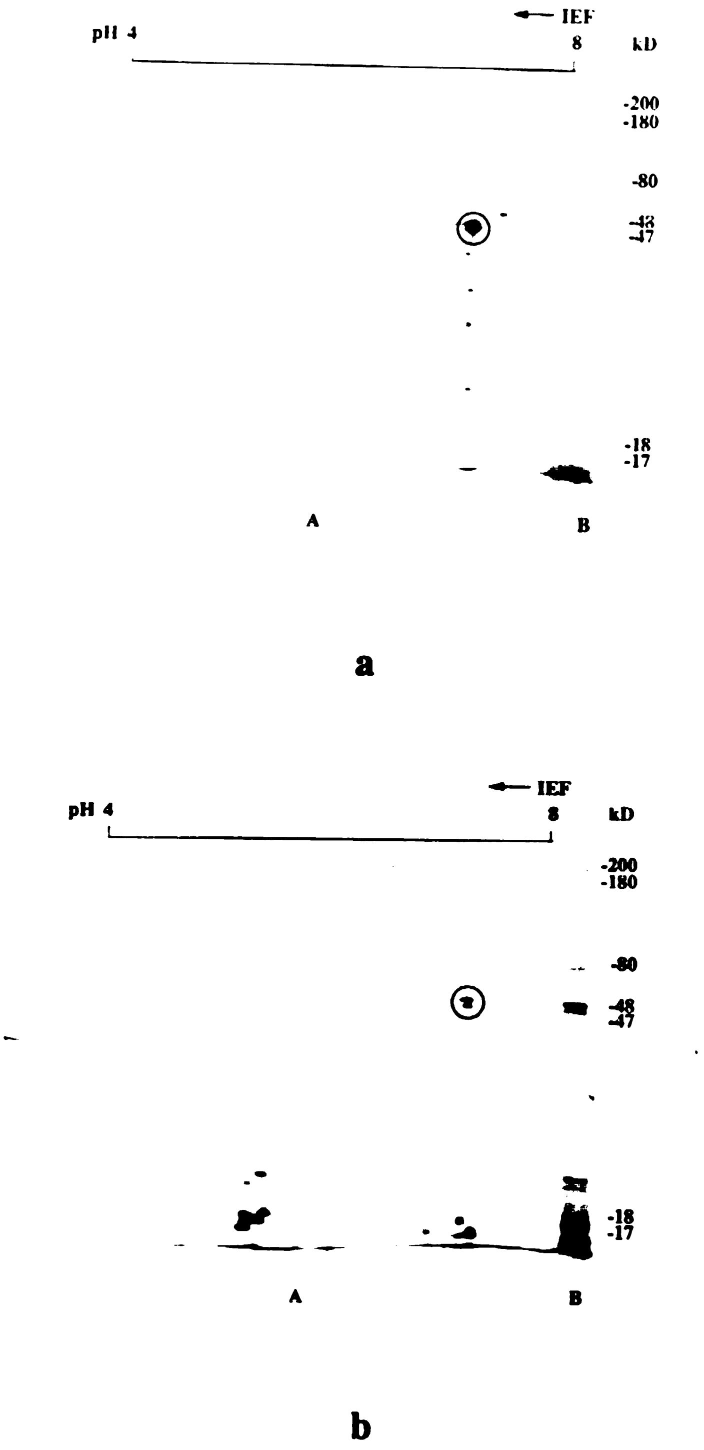

IEF
pH 4
8
kD
-200
-180
-80
-48
-47
-18
-17
A
B
a
IEF
pH 4
8
kD
-200
-180
-80
-48
-47
-18
-17
A
B
b

Figure 7

Table 1. Mole percent amino acid composition of COR polypeptide sequence

Amino Acid		Mol%
Gly	G	26.85
Ala	A	7.67
Val	V	1.79
Leu	L	2.30
Ile	I	2.05
Ser	S	0.26
Thr	T	15.86
Cys	C	0.00
Met	M	2.30
Asp	D	2.56
Asn	N	1.28
Glu	E	5.37
Gln	Q	6.91
Arg	R	0.77
Lys	K	6.91
His	H	11.00
Phe	F	0.00
Tyr	Y	3.07
Trp	W	0.00
Pro	P	3.07

Figure 8. Hydrophy profile of the deduced amino acid sequence for COR39. The plots are according to Kyte and Doolittle (1978) using a window of nine amino acid. Negative values indicated hydrophilicity.

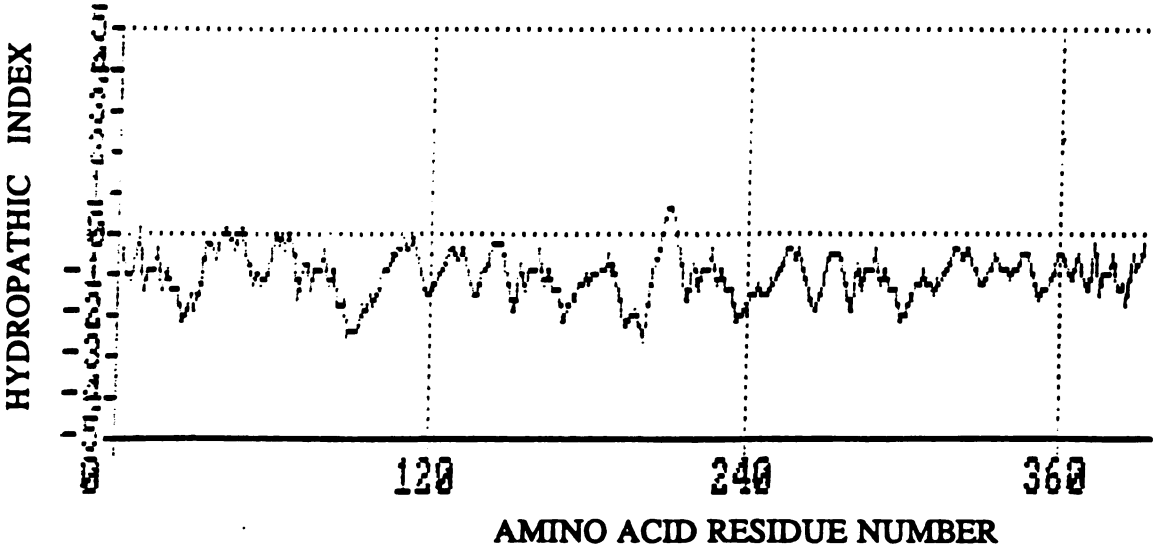
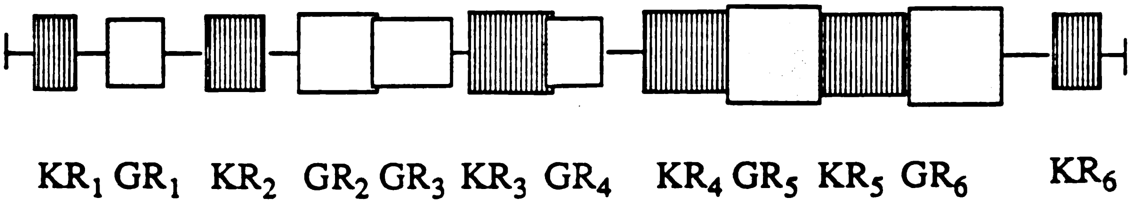


Figure 8

Figure 9. The repeating units of *cor39*. KR: lysine-rich repeat (▥), GR: glycine-rich repeat (□). The lysine-rich and glycine-rich reats are numbered as in Fig.6.



Lysine-rich Repeat	
KR1	AGEKKGIMEKIKEKLPGGH
KR2	AGEKKGVMENIKDKLPGGH
KR3	TGEKKGLMENIKEKLPGGHGDHQQTAG
KR4	GTGEKKGVMENIKDKLPGGHGDHQQTGG
KR5	GTGEKKGVMENIKEKLPGGHGDHQQTGG
KR6	GEKKSLMDKIKDKLPGQH
Glycine-rich Repeat	
GR1	GGAYGQQGHAG
GR2	GGHYGQQGHAGTATHGTPATAGTY
GR3	GTYGQQGHTGTATHGTPATGGTY
GR4	GTYGQQGHVGTGTH
GR5	GGTYGQQGHTGTATHGTPAGGGTYEQHGHTGMTGTGT
GR6	GGAYGQQGHTGTATHGTPAGGGTYGQHATGMTGTET

Figure 9

Table 2. Comparison of lysine-rich repeat of COR39, COR47 and Lea proteins.

Polypeptide	Lysine-rich Repeat
COR39 KR$_1$	AGEKKGIMEKIKEKLPGGH
KR$_2$	AGEKKGVMENIKDKLPGGH
KR$_3$	TGEKKGLMENIKEKLPGGHGDHQQTAG
KR$_4$	GTGEKKGVMENIKDKLPGGHGDHQQTGG
KR$_5$	GTGEKKGVMENIKEKLPGGHGDHQQTGG
KR$_6$	GEKKSLMDKIKDKLPGQH
COR47	EDKKGLVEKIKEKLPGHHD
B17	RRKKGLKDKIKEKLPGGHGD
B18	RRKKGIKEKIKEKLPGGHGD
RAB21	RRKKGIKEKIKEKLPGGNK
Conserved Consensus Sequence*	KKGB-XZIKXKLPGGH

*nonstandard abbreviations: B,I/L/V; X,E/D; Z,K/N.

Talbe 3. Comparison of the glycine-rich repeat of COR39, RAB21, and barley dehydrin B17 and B18.

Polypeptide		Glycine-rich Repeat
COR39	GR$_1$	GGAYGQQGHAG
	GR$_2$	GGHYGQQGHAGTATHGTPATAGTY
	GR$_3$	GTYGQQGHTGTATHGTPATGGTY
	GR$_4$	GTYGQQGHVGTGTH
	GR$_5$	GGTYGQQGHTGTATHGTPAGGGTYEQHGHTGMTGTGT
	GR$_6$	GGAYGQQGHTGTATHGTPAGGGTYGQHATGMTGTET
B17		GGTYGQHGHTGMTGTG
		GGTYGQQGHTGMTGT
B18		GYGQQGTGMAGT
		GGTYGQQGHTGMTGMGA
		GTYGQQGHTGMAGTGA
		GGTYGQQGHTGMTGTGM
		GGTYGQQGHTGMTGTGM
RAB21		GGAYGQQGHGTGMTTGT
Conserved Consensus Sequence		G-YGQQGH--

Figure 10. Time course of accumulation of *cor39* transcripts in plants treated with low temperature (2° C): A, 0 hour; B, 2 hours; C, 4 hours; D, 6 hours; E, 8 hours; F, 10 hours; G, 12 hours; H, 24 hours. 15 ug of total RNA was fractionated on a formaldehyde agarose gel, transferred to a Nytran membrane and hybridized with ^{32}P-labeled pWG1 insert.

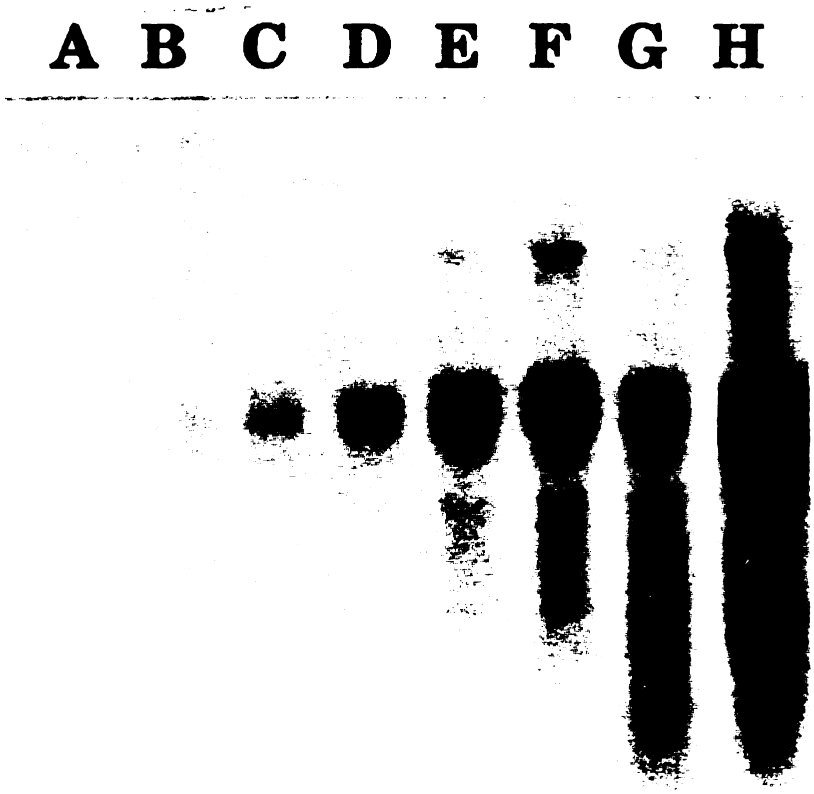


Figure 10

orientation (pWG1) yielded major polypeptides of about 48 and 47 kD that were boiling-stable and had pIs of approximately 7.5 (Figure 7a). *In vitro* transcription/translation of pWG2 did not yield any translation products (data not shown). *In vitro* translation of poly (A^+) RNA isolated from cold acclimated plants also result in the synthesis of 48 and 47 kD boiling-stable polypeptides with pIs of about 7.5 (Figure 7b). Presumably these are the same polypeptides detected in the *in vitro* transcription/translation reaction of pWG1. Thus, it would appear that pWG1 has the entire open reading frame for the gene it represents. This gene was designated *cor39* and the polypeptide it encodes, COR39.

Analysis of COR39

The COR39 polypeptide is glycine-rich (27%), and contains a high proportion of threonine (16%) and histidine (11%). but neither cystine nor tryptophan (Table 1). The hydropathy profile of the polypeptide indicates it is hydrophilic (Figure 8), the mean hydropathic index of the polypeptide is -1.1. The majority of COR39 is composed of two repeating sequences (Figure 6 and 9). The first is a lysine-rich sequence designated KR, that occurs six times. The repeat is imperfect with variation in amino acid composition and number. Nearly identical lysine-rich repeats occur in COR 47 (Gilmour *et al.*, 1991) and the group II family of LEA proteins (Dure *et al.,* 1989). In Table 2, the lysine-rich repeat of COR39, COR47, the RAB21 LEA protein of rice (Mundy and Chua, 1988) and the B17 and B18 dehydrins of barley (Close *et al.*, 1989) are presented. A comparison of the sequence indicates a conserved consensus sequence of KKG(I/LV)-(E/D)(K/N)IK(E/D)KLPGGH.

The second amino acid repeat in COR39, designated GR, is glycine-rich (Figure 6 and 9). It too is an imperfect repeat, varying in sequence length and composition. Closely related glycine-rich repeats occur in the barley dehydrins and LEA protein RAB21 (Table 3). A comparison of these sequences indicates a conserved consensus sequence of G-YGQQGH.

A TFASTA search of the GenBank/EMBL database (release 68.0 June, 1991), resulted in barley dehydrin B17 having the highest score for sequence similarities with COR39 while the barley dehydrin B18 comes in second. Other LEA proteins were also on the list. No proteins other than LEA proteins were found to have significant sequence similarity with COR39.

Figure 11.Time course of deaccumulation of *cor39* transcripts in plants transferred back to normal growth temperature (deacclimated) after treatment at 2° C for 3 days. A, plants treated at 2° C for 3 days; B, deacclimated for 2 hours; C, deacclimated for 4 hours; D, deacclimated 6 hours; E, deacclimated for 8 hours; F, deacclimated 10 hours; G, deacclimated 12 hours; H, deacclimated for 1 day; I deacclimated for 1 week. 15 ug of total RNA was fractionated on a formaldehyde agarose gel, transferred to a Nytran membrane and hybridized with ^{32}P-labeled pWG1 insert.

Figure 11

Figure 12. The effect of temperature treatment on accumulation of *cor*39 transcripts in plants. A, Plants were grown at 20° C for two weeks; B-F, plants were treated at 18, 16, 14, 12 and 2° C, respectively, for one week. 15 ug of total RNA was fractionated on a formaldehyde agarose gel, transferred to a Nytran membrane and hybridized with ^{32}P-labeled pWG1 insert.

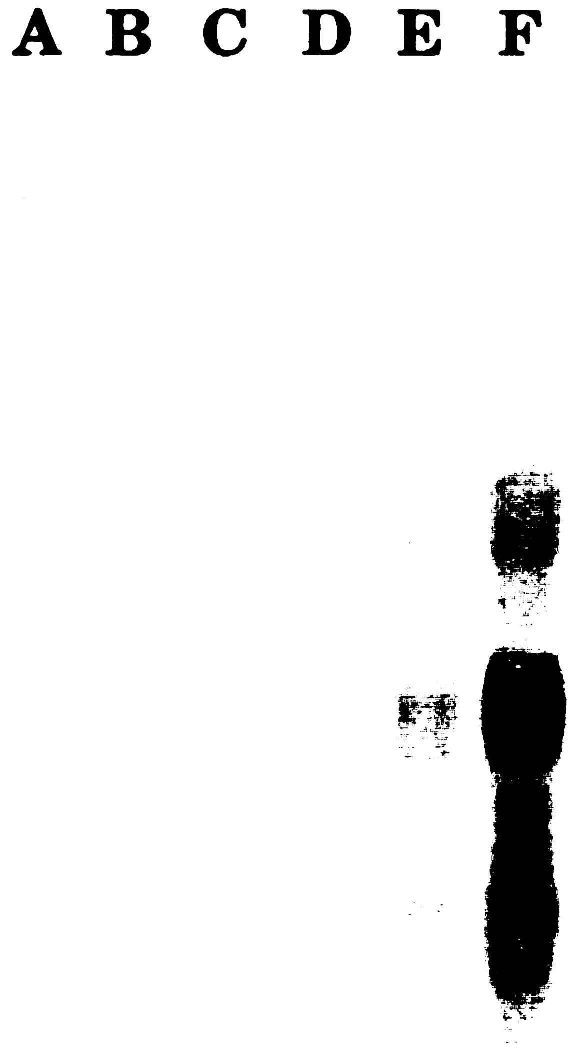


Figure 12

Figure 13. Tissue specificity of *cor*39 transcripts accumulation in plants cold acclimated at 2 ° C for 1 week. A and B, cold acclimated roots (two independent experiments); C, nonacclimated roots; D and E, cold acclimated crowns (two independent experiments); F, non-acclimated crowns; G and H, cold-acclimated leaves (two independent experiments); I, nonacclimated leaves. 15 ug of total RNA was fractionated on a formaldehyde agarose gel, transferred to a Nytran membranes and hybridized with ^{32}P-labeled pWG1 insert.

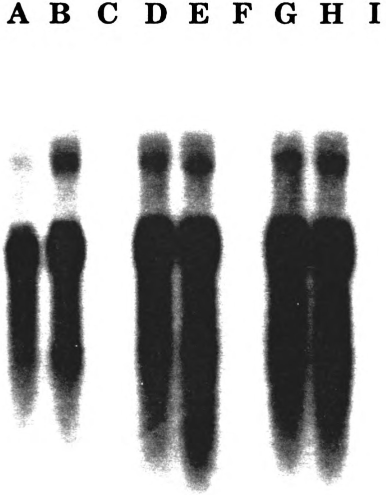


Figure 13

Figure 14. Genomic Southern analysis of *cor*39. A, genomic DNA was digested with *EcoR*I; B, Genomic DNA was digested with *BamH*I. 20 ug of genomic DNA fractionated on a 1% agarose gel, transferred to a Nytran membranes and hybridized with ^{32}P-labeled pWG1 insert.

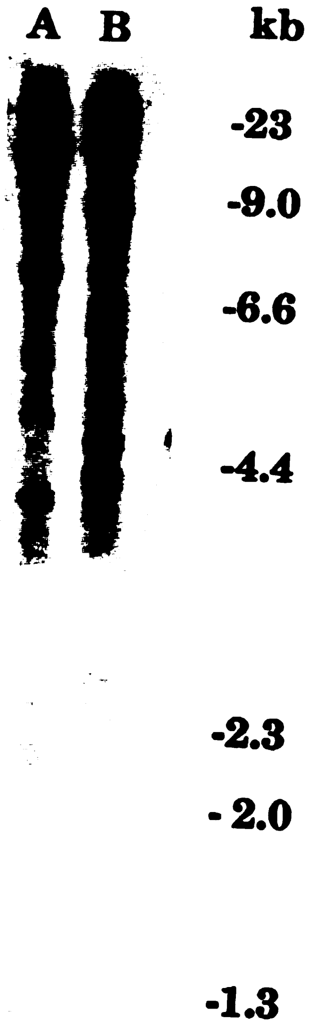


Figure 14

Figure 15. Accumulation of *cor*39 transcripts by ABA treatment. A, plants cold-acclimated at 2° for 3 days; B, control plants; C, plants treated with ABA for 2 hours; D, plants treat with ABA for 4 hours; E, plants treated with ABA for 6 hours; F, Plants treated with ABA for 8 hours. 15 ug of total RNA was fractionated on a formaldehyde agarose gel, transferred to a Nytran membrane and hybridized with ^{32}P-labeled pWG1 insert.

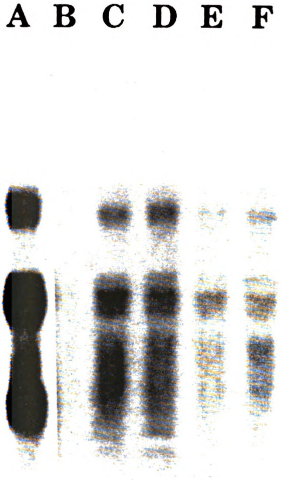


Figure 15

Figure 16. Accumulation of *cor*39 transcripts by drought stress treatment. A, control plants (relative water content 93%); B, water-stressed plants (relative water content 55%); C, plants cold-acclimated at 2°C for 3 days. 15 ug of total RNA was fractionated on a formaldehyde agarose gel, transferred to a Nytran membrane and hybridized with ^{32}P-labeled pWG1 insert.

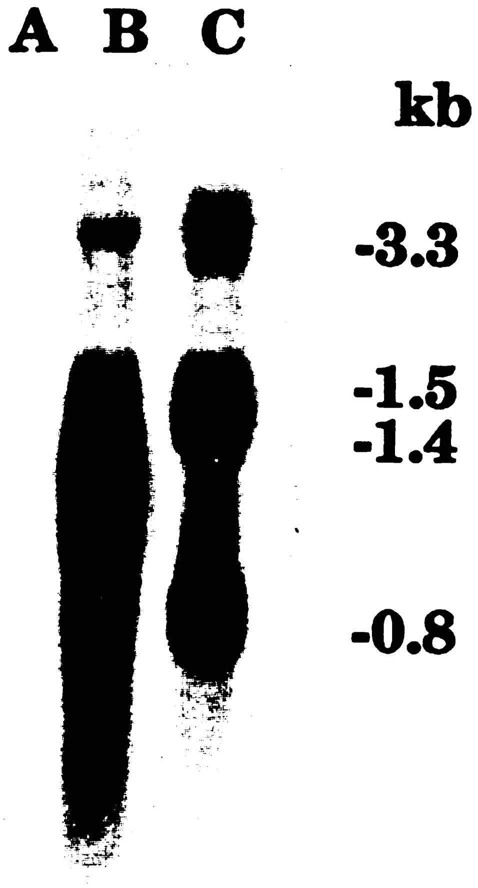


Figure 16

Figure17. Northern analysis of *cor*39 transcripts in fresh dry seed and seedlings of *T. acestivum* cv Augusta. A, fresh dry seed: B, seedlings after 3 day germination; C, acclimated leaves of Winoka (2° C, one week). 15 ug of total RNA was fractionated on a formaldehyde agarose gel, transferred to a Nytran membrane and hybridized with ^{32}P-labeled pWG1 insert.

Figure 17

Expression of *cor39* in Response to Low Temperature

Northern analysis (Figure 10) indicated that *cor*39 transcripts started to accumulate after wheat plants had been treated with low temperature (2 ° C) for 2 hours, and that they continued to increase in concentration up to about 12 hours. The *cor39* transcripts remained at high levels for as long as the plants were kept at low temperature (up to three weeks, Figure 3). When cold acclimated plants were transferred back to normal growth temperature for 2 hours, the level of the *cor39* transcripts markedly decreased and by about 4 hours they returned to that of nonacclimated plants (Figure 11). The threshold temperature at which *cor*39 transcripts markedly increased was 12° C (Figure 12). cor*39* transcripts were detected in roots, crowns and leaves of acclimated plants (Figure 13).

Southern Analysis

Hybridization of the pWG1 insert with a Southern blot of total genomic wheat DNA resulted in the detection of multiple bands (Figure 14). These data indicated that either *cor39* or related genes were present at multiple copies in the wheat genome.

Expression of *cor39* Gene in Response to ABA and Water Stress

It has been reported that exogenous application of ABA at normal growth temperature can result in increased freezing tolerance in many plants including wheat and rye (Chen and Gusta, 1983; Cloutier, 1983; Lang *et al.*, 1989). Therefore, it was of interest to determine whether the expression of *cor39* responded to ABA. The data indicate that it did (Figure 15): *cor*39 mRNAs accumulated in plants sprayed with 100 uM of ABA solution for two hours and remained at the some level of accumulation after 8 hours after the spray, even the levels of accumulation decreased after 6 hours treatment. The level of *cor39* transcripts in the ABA treated plants, however, was much lower than that in cold acclimated plants (Figure 15). Four independent experiments were conducted with similar results.

The expression of *cor39* was also responsive to drought stress (Figure 16). When the relative water content of plants fell to about 55%, the level of *cor39* mRNAs increased dramatically.

cor39 Transcripts in Fresh Dry Seed

Transcripts of *lea* genes accumulate to a high level in late embryo development and remain at high levels in fresh dry seeds (Dure *et al.*, 1989). Therefore, it was of interest to determine if *cor39* transcripts were also present at high level in fresh dry seeds. Because I did not have fresh dry seed for winter wheat Winoka, I isolated total RNA from fresh dry seeds (about one month after harvest) of Augusta, another winter wheat variety. In addition, RNA was isolated from the seedlings of these seeds after germination for three days. The result of Northern analyses indicate that cor*39 t*ranscripts that accumulated upon cold acclimation were not present at high level in fresh dry seed, but that transcripts of about 1.3 kb were present. The transcripts decreased dramatically after three days of germination.

Discussion

The amino acid sequence of COR39 was deduced from the nucleotide sequence of the *cor*39 cDNA clone pWG1. The sequence data indicate that COR39 is related to Group II LEA proteins, and like LEA proteins, COR39 is hydrophilic and boiling-stable. COR39 has the lysine rich repeats that the Group II LEA proteins have (Dure *et al.*,1989). In addition, COR39 has the glycine-rich semi-conserved repeats which the barley dehydrins B18 and B17 have (Close *et al.*, 1989). Also, COR39, like the LEA proteins and dehydrins, is responsive to both ABA and droughts stress. However, COR39 has features which are different from LEA proteins and dehydrins. First, it does not have a seven residue serine cluster which all the Group II LEA proteins have. It is reported that the serine cluster is the site for protein phosphorylation of LEA protein RAB17 (Vilaardell *et al.*, 1990). Second, COR39 has six lysine-rich repeats while all the LEA proteins including the dehydrins from barley and maize, and the salt-stress related polypeptides TAS14 of tomato (Godoy *et al.* 1990) have only two lysine-rich repeats. Interestingly, COR47, the cold-regulated polypeptide from *Arabidopsis*, has at least three lysine repeats (Gilmour *et al*, in press).

Given the common features that COR39 shares with LEA proteins, the question is raised whether COR39 has the same function as LEA proteins and dehydrins. The role of LEA proteins is unknown, but they are hypothesized to help cells tolerant water stress (Close *et al.*, 1989; Dure *et al.*, 1989). Interestingly, it is known that extracellular ice formation causes cell dehydration. Thus, freezing tolerance must include water-stress tolerance.

Indeed, it has been reported that water stress can increase the freezing tolerance of certain plants including wheat and rye (Cloutier,1983). It therefore seems reasonable to hypothesize that freezing and drought tolerance might involve related genetic mechanisms and gene products. These gene products, which are very hydrophilic polypeptides, all might have the general function of protecting cells from water stress, no mater whether the water stress is from freezing or drought stress. The expression of these genes may be regulated by a common signal warning of water stress. This signal could be ABA, since ABA increases in water-stressed plants (Close *et al.*, 1989; Dure *et al.*, 1989) and it has been reported that ABA can increase the tolerance of plants to both drought and freezing stress (Chen and Gusta, 1983; Cloutier, 1983; Lang *et al.*, 1989). Further, ABA can induce the expression of these water-stress or cold-regulated genes. However, the *cor* and *lea* gene products may also have specific role in freezing tolerance or desiccation stress such as that occurs during embryogenesis. More studies about the function and regulation of the COR and LEA polypeptides are required in order to better understand their relationship.

The 1.3 kb transcript, which is present in the fresh dry seeds but not in the cold acclimated plant, can originate from *cor39*, representing different sites of transcript initiation or different processing events. Alternatively, they can be also transcribed from gene related to *cor39*. Additional experiment will be required to distinguish between these possibilities.

The levels of *cor39* transcripts decrease after 6 hours ABA treatment compared to the 2 hours ABA treatment. The possible explanation is ABA level in plant decreases after 6 hours treatment, because ABA is easy to be metabolized in plants.

cor39 transcripts were found to accumulate when plants were treated at 12° C for three weeks, but the level of accumulation was much lower than in plants treated at 2° C (Figure 12). Interestingly, plants gained some freezing tolerance when they were treated at 12 ° C (Figure 1), but the freezing tolerance was much weaker when compared to that of plants treated at 2° C. These results suggest the possible positive correlation of the level of acclimation of *cor39* transcripts with level of freezing tolerance. However, there is no such correlation in the early stage of cold acclimation. It should be noted that the accumulation of *cor*39 transcripts starts after the plants have been treated with low temperature for four hours and that accumulation reaches a peak after one day (Figure 10). At this early stage the accumulation of these transcripts is not correlated to freezing tolerance; the freezing tolerance increases only from -5°C to -8° C after one week of 2° C treatment, while three weeks of treatment increases the freezing tolerance from -5° C to -14° C. One possible explanation could be that although the transcripts are accumulating with hours of cold treatment, the gene products might take days to accumulate. We do not know if COR39 is synthesized *in vivo* with similar kinetics to its transcripts. We also do not know if there is any translational or posttranslational control of these genes. Further, cold acclimation is a very complex process, involving molecular, biochemical, and physiological changes. The COR proteins may play critical roles in freezing tolerance, but other biochemical and physiological changes, that develope slowly, might also be required for attain maximum freezing tolerance. Studies directed toward determining the exact function of the COR proteins and their relationship to low temperature survival are needed.

The COR39 open reading frame encodes a polypeptide of molecular weight 39.1 kD, pI=7.48. Thus, there is an 8 kD difference between the molecular weight deduced from the pWG1 sequence and that predicted from its mobility in SDS-PAGE. It has also been reported in other LEA proteins that there is a difference between the molecular weight data deduced from DNA sequence and that predicted from the mobility in SDS-PAGE. For RAB21, the predicted molecular weight from SDS-PAGE is 21 kD, while the deduced molecular weight is 16.5 kD (Mundy and Chua 1988). For RAB17, the apparent molecular weight is 23 kD, while the deduced molecular weight is 16.5 kD. TAS14 is a cDNA clone isolated from tomato that represent a gene that responds to salt stress and ABA but not low temperature (Godoy *et al.*, 1990). Its deduced polypeptide sequence indicates it is related to Group II LEA protein. Its apparent molecular weight in SDS-PAGE is 16 kD while its deduced molecular weight is 13.9 kD. In all of these cases, the proteins are very hydrophilic. Perhaps this biophysical feature explains why they all have a difference between the deduced molecular weight and predicted molecular weight on SDS-PAGE.

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Appendix

Appendix

Correlation of Wheat Freezing Tolerance with Expression of Cold-Regulated (*cor*) Genes.

In order to determine whether there was a relationship between freezing tolerance and the expression of cold-regulated (*cor*) genes, I exam *cor39* expression in another winter wheat variety, Genesee, and two spring wheat varieties. All the experiments were conducted in the same way as described as Chapter II. The following are some preliminary results:

1. Comparison of Freezing Tolerance of Different Varieties:

Variety	LT_{50}		
	20° C[1]	12°C[2]	2° C[3]
Winoka	-5° C	-10° C	-14° C
Genesee	-5° C	-7° C	-11°C
Spring wheat$_1$ (S_1)	~	~	~
Spring wheat$_2$ (S_2)	-5°C	~	-9° C

"~" did not test. 1, nonacclimated plants; 2, plants were cold-acclimated at 12° C for 3 weeks; 3, plants were cold acclimated at 2° C for three weeks.

Among the three varieties I tested, Winoka was the most freezing tolerant, Genesee was next, and Spring wheat$_2$ was third.

2. Changes in Gene Expression Associated with Cold Acclimation.

Experiments were performed to determine whether the transcripts encoding boiling-stable polypeptides accumulated during cold acclimation. The data indicate that they do (Figure 18). However, at 12° C, the accumulation of these polypeptides were stronger in Winoka than in Genesee (Figure 18, Lanes A and C). At 12° C, the levels of these boiling-stable polypeptides were not markedly increased in Spring wheat$_1$. At 2°C, the levels of these boiling-stable proteins in Winoka were higher than that in Genesee and Spring wheat$_1$(Figure 18, Lanes B, D, F, H, J). At both 12 and 2° C, in both Genesee and Spring wheat, there was no, or very low, accumulation of the 180 kD cold-induced boiling-stable polypeptide.

3. Northern Analysis

Similar to the cold-induced boiling-stable polypeptides, at 12°C, the levels of *cor39* transcripts were much higher in Winoka than that in Genesee (Figure 20, Lanes G and I). For Spring wheat$_1$, there was very low accumulation of *cor39* transcripts (Figure 20, Lane C). At 2° C, the levels of *cor39* transcripts in winoka was much higher than that of Genesee, Spring wheat$_1$ and Spring wheat$_2$ (Figure 20, Lanes B, D, F, H, J). Comparing Winoka and the other three varieties, the major difference in the pattern of accumulation of *cor39* transcripts was the upper band (about 3.3 kb). The levels of the upper band of *cor39* transcripts were much lower than that of Winoka.

4. Discussion

Winoka is the most freezing tolerant variety among the three varieties tested, Genesee is next and Spring wheat$_2$ is third; I did not test Spring wheat$_1$. The data above indicate that there is a possible positive correlation of accumulation of boiling-stable polypeptides, accumulation of *cor39* transcripts and freezing tolerance. The major difference in the pattern of accumulation of boiling-stable polypeptides between Winoka and the other three varieties is the 180 kD polypeptide.The major difference in the pattern of accumulation of *cor39* transcripts is in the upper band. The relationship of this upper band and the 180 kD boiling-stable polypeptide is not known. It appears from these preliminary data that there is a possible positive correlation between the freezing tolerance and the accumulation of *cor39* transcripts, especially the upper bands. However, I did not collect these data quantitatively. Therefore, more studies are needed in order to prove these correlations

Other Wheat *cor* cDNA Clones Related to pWG1

I have used pWG1 to screen the wheat cDNA library to isolate genes related to *cor39*. Five clones showed homology to pWG1. These clones were purified and analyzed further.

1. pG1:

The insert is about 0.8-1 kb The gene represented by this insert is cold-regulated. One *Eco*RI site is lost in the polylinker of the plasmid; the insert can be cut out by *Eco*RI plus *Bam*HI.The two ends of this clone have been sequenced for 100-200 bp. The sequence data showed that pWG1 and pG1 are not identical.

2. pG2:

This clone contained three inserts, about 0.8, 0.7 and 0.6 kb. The gene represented by the 0.6 kb insert is cold-regulated, but not the other two inserts. The two ends of this clone were sequenced for about 100 bp. The data showed that pWG1 and pG2 are not identical.

3. pG4

DNA sequencing of the ends of pG4 and restriction analysis indicate that it is identical to pWG1.

4. pG5 and pG6

Both pG5 and pG6 had two inserts of about 0.6 and 0.7 kb. The two inserts had some common restriction sites. Probably pG5 and pG6 are identical. The genes represented by the two inserts in pG6 are cold -regulated. The inserts in pG5 were not tested to see if they represented cold-regulated genes.

Non of the experiments mentioned above were repeated.

Figure 18. SDS-PAGE analysis boiled products of *in vitro* translation products of Poly (A^+) RNA isolated from different varieties acclimated at different temperatures. A, Genesee acclimated at 12°C for 3 weeks; B, Genesee acclimated at 2° C for three weeks; C, Winoka acclimated at 12°C for 3 weeks; D, Winoka acclimated at 2° C for 3 weeks; E, Spring wheat $_1$acclimated at 12°C for 3 weeks; F, Spring wheat$_1$ acclimated at 2°C for 3 weeks; G, Winoka grown at 20 ° C for 2 weeks H, Winoka acclimated at 2°C for 3 weeks; I, Spring wheat$_1$ grown at 20° C for 2 week.; J, Spring wheat$_1$ acclimated at 2°C for 3 weeks;

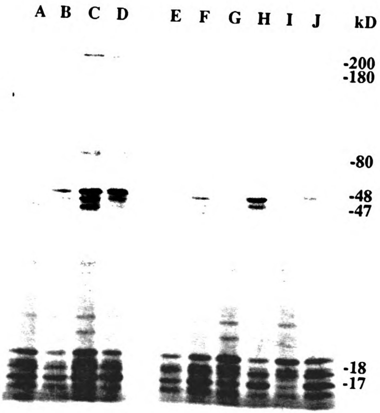

Figure 18

Figure 19. The effect of temperature treatment on accumulation of *cor*39 transcripts in different varieties. A, Spring wheat $_2$grown at 20° C for 2 weeks; B, Spring wheat $_2$ acclimated at 2°C for 3 weeks; C, Spring wheat $_1$ acclimated at 12°C for 3 weeks; D, Spring wheat $_1$acclimated at 2°C for 3 weeks; E, Spring wheat$_1$ grown at 20° C for 2 week.; F, Spring wheat$_1$ acclimated at 2°C for 3 weeks; G, Winoka acclimated at 12°C for 3 weeks; H, winoka acclimated at 2° C for three weeks; I, Genesee acclimated at 12°C for 3 weeks; J.Gensee acclimated at 2° C for three weeks.

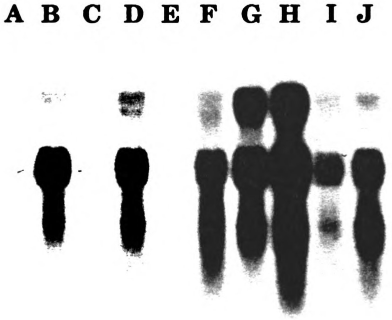

Figure 19