This is to certify that the

dissertation entitled

Cloning, characterization and regulation of four
cold-induced genes from Arabidopsis thaliana

presented by

David P. Horvath

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Genetics

Major professor
Michael Thomashow

Date March 3, 1993

Cl

c

Cloning, characterization and regulation of four cold-induced genes from *Arabidopsis thaliana*

By

David P. Horvath

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirments
for the degree of

DOCTOR OF PHILOSOPHY

Program in genetics

1993

C

Co

plants to

is known

response

changes

genes fr

four dis

low ten

cor ger

genes

the gro

cor78,

level,

Studie

demo

impli

pathv

Abstract

Cloning, characterization and regulation of four cold-induced genes in *Arabidopsis thaliana*

By

David P. Horvath

Considerable work has been done to characterize the physiological responses of plants to low temperatures and during the process of cold-acclimation. However, little is known about how plants sense cold and alter their pattern of gene expression in response to low temperatures. In order to gain insight into the mechanisms involved in changes in gene expression during cold acclimation, it was decided that cold-regulated genes from *Arabidopsis thaliana* should be isolated and studied. cDNAs representing four distinct cold-regulated (*cor*) genes were cloned. The *cor* genes are all induced by low temperatures, drought stress, and by exogenous application of abscisic acid. The *cor* genes are not induced, (or are poorly induced) by heat shock and wounding. These genes demonstrate a threshold of induction at 10°C and are expressed throughout all of the growing stages of the plant. Work focusing on one of these *cor* genes (designated *cor78*) has demonstrated that this gene is regulated primarily at the transcriptional level, and it is expressed throughout most of the plant in response to low temperatures. Studies on the kinetics of the induction and inactivation of the *cor78* promoter has demonstrated that this gene has a half-life of approximately 35 minutes. Finally, the implications of all of these results concerning the possible signal transduction pathway(s) that may function in the regulation of this gene are discussed.

I wo
throughout t
Ravindra Ha
Wilhelm, Na
(scientific ar
efforts and p
manuscript I
given to Dr.
short notice
committee.
during the lo
quests. Fina
Levanthal, ar
from them ea

ACKNOWLEDGMENTS

I would like to thank Dr. Mike Thomashow for his support, help, and ideas throughout this project. I would also like to thank my co-workers Sarah Gilmour, Ravindra Hajela, Tim Lynch, Brett McLarney, Tod Cotter, Stokes Baker, Kathy Wilhelm, Nancy Artus and Deane Lehmann for their encouragement and discussions (scientific and otherwise). Special thanks goes to Sarah Gilmour for her tremendous efforts and patience in the critical reviews of this dissertation and every other manuscript I have written since I have known her. Also, special thanks should be given to Dr. Lee MacIntosh and Dr. Rebecca Grumet for joining my committee on short notice and to Dr. Jerry Dodgson for his help as the lone original member of my committee. I am also grateful to my wife and son for their patience and understanding during the long periods of time that I have spent away from them during my scientific quests. Finally, I would like to acknowledge Dr. Terry Mathews, Dr. Mark Levanthal, and Dr. Jeff Bennetzen for the inspiration and encouragement that I received from them early in my scientific career.

TABLE OF CONTENTS

APPENDIX

Table 2.

Table 2

Table 3

LIST OF TABLES

LIST OF FIGURES

page

Figure

Figure

Figure

ABA: ab

°C: degr

CaMV: c

cDNA: c

CIM: cal

DNA: de

DS: doub

DTT: dit

EtOH: et

GBF: G-

GUS: bet

Lt_{50}: leth

NEB: Ne

Nos: nopa

PCI: pher

PCR: pol

RNA: rib

SDS-PAC
ele

SIM: sho

URE: ups

UTP: uric

UTR: unt

List of abbreviations

ABA: abscisic acid

°C: degrees centigrade

CaMV: cauliflower mosaic virus

cDNA: copy deoxyribonucleic acid

CIM: callus inducing media

DNA: deoxyribonucleic acid

DS: double stranded

DTT: dithiothreitol

EtOH: ethanol

GBF: G-box binding factor

GUS: beta glucuronidase

Lt$_{50}$: lethal temperature 50%

NEB: New Engalnd Biolabs

Nos: nopalin synthase

PCI: phenol/chloroform/iso-amyl alcohol

PCR: polymerase chain reaction

RNA: ribonucleic acid

SDS-PAGE: sodium dodecyl sulfate- poly acrylamide gel electrophoresis

SIM: shoot inducing media

URE: upstream regulatory element

UTP: uridine 5'-triphosphate

UTR: untranslated region

Chapter 1
LITERATURE REVIEW

I. INTRODUCTION

Plants that are indigenous to temperate regions of the earth are often exposed to extremes in low temperature. Unlike their animal counterparts, plants are unable use avoidance as a means of countering this type of stress. Thus, it is not surprising that some plants have evolved means by which they can sense temperature drops and alter their biochemistry in order to prevent or minimize systemic damage caused by freezing. In fact it is known that many plants, if first subjected to low but non-freezing temperatures, are able to become more tolerant to freezing stress (see Levitt, 1980; Sakai and Larcher, 1987; Thomashow, 1990). This process is called cold acclimation.

II. COLD STRESS PHYSIOLOGY

Much is known about the changes in plant biochemistry that occur during the cold acclimation process. Many plants show a build up of small soluble molecules such as proline and sugars (Sakai and Larcher, 1987). Often, changes in the composition of membrane lipids also accompany the plant's response to the cold (Steponkus and Lynch, 1989). Finally, it has been shown that there are specific changes in the protein profile of plants that have undergone cold acclimation (Levitt, 1980; Thomashow, 1990; Guy, 1990), and there is evidence to suggest that protein synthesis is necessary for the cold acclimation process to occur (Weiser, 1970). Although it is known that all these changes occur, with the exception of a few cases (such as changes in the phospholipid composition of the plasma membrane (Steponkus 1989)), it has yet to be demonstrated that any are directly responsible for bringing about a greater tolerance to freezing temperatures in the plant.

Arabidopsis thaliana was chosen as a model system for studying cold acclimation due to its many advantages for molecular study (Meyerowitz, 1989), and because earlier work on this plant has demonstrated that it is capable of acclimating to cold temperatures (Gilmour *et al.*, 1988). Studies using electrolyte leakage to determine the relative cellular damage caused by a freeze-thaw cycle demonstrated that leaf tissue from acclimated *Arabidopsis* plants leaked less than 50% of their electrolytes at a temperature of -8°C. When compared to non-acclimated tissue, which reached the same level of leakage at a temperature of -3°C, this represented a significant change in the ability of the acclimated tissue to survive freezing. However, since the leakage of electrolytes can be the result of relatively minor or non-permanent damage to the cell membrane, these electrolyte leakage experiments may not provide an accurate representation of the true survivability of an *Arabidopsis* plant to a freeze-thaw cycle. Acclimation studies using growth after a freeze-thaw cycle as an indication of incurred damage have shown that both non-acclimated and acclimated plants are capable of withstanding lower temperatures (-5°C and -20°C, respectively) than one would expect given the results of electrolyte leakage experiments (Singh, unpublished results).

To better understand what a plant must do in order to acclimate, one needs to understand the physical nature of the damage caused by a freeze-thaw cycle. As the temperature drops below 0°C, water in the extracellular spaces begin to freeze. This has the effect of lowering the water potential outside the cell, and thus water moves into the extracellular spaces with the lower potential and the cell becomes dehydrated. As the cell thaws, water moves back into the cell at a rate that is dependent on the rapidity of the temperature increase (Gordan-Kamm and Steponkus, 1984). The stress caused by this freezing induced dehydration and subsequent rehydration is a major cause of damage to plant cells that are exposed to temperatures between $0^{o}C$ and $-10^{o}C$ (Steponkus, 1984).

Studies using isolated protoplasts from rye as a model system, have shown that nonacclimated cells form intracellular vesicles in response to the decrease in cell volume caused by freezing induced dehydration, and that when the cell rehydrates upon thawing these vesicles are not reincorporated back into the membrane fast enough and the cell bursts. In acclimated cells, the cell membrane forms extracellular extrusions that are readily reincorporated into the membrane upon thawing (Gordon-Kamm and Steponkus, 1984). Moreover, there is evidence that the ability to form these extracellular extrusions is the result of specific changes in the phospholipid composition of the acclimated cell's membrane (Steponkus and Lynch, 1989). Thus, the acclimating cell may increase the production or activity of enzymes that can effect changes in the phospholipid composition of the membrane.

Freeze-thaw induced cell lysis is not the only mechanism by which a cell can be damaged during freezing. Many proteins may also be susceptible to the dehydration effects experienced during freezing (Brandts, 1967), as interactions with water are required to maintain their three dimensional form. When water in the cell becomes limiting, these proteins are likely to become denatured and form aggregates which precipitate out of solution. Therefore, chaperonin-like proteins (Gatenby, 1992) may be induced in cells acclimating to the cold in order to protect exposed hydrophobic surfaces. Alternatively, some highly charged proteins can order the water molecules around membranes and other proteins (Reviewed in Carpenter and Crowe, 1988). This may have the effect of decreasing the energy potential of the water molecules in their immediate area (Reviewed in Carpenter and Crowe, 1988). Since water will move from areas of higher water potential to areas with lower water potential, the stabilizing effect of these charged proteins may help maintain a layer of water around other proteins and membranes (Reviewed in Carpenter and Crowe, 1988).

Since freezing stress often involves severe dehydration, it is not surprising that a number of parallels to drought stress have been documented. In both stresses, the levels of the plant hormone abscisic acid (ABA) have been shown to become elevated (albeit only transiently in some cold-treated plants) (Daie and Campbell, 1981; Chen *et al.*, 1983). It has been shown that in some plants, both drought stress and the exogenous application of ABA can increase freezing tolerance (Chen and Gusta, 1983; Gusta, 1983; Lang *et al.*, 1989). Finally, many of the cold-regulated (*cor*) genes which have been cloned, are also induced by both drought and ABA (Mohapatra *et al.*, 1988; Hejela *et al.*, 1990). Such observations have led to the development of a model where some environmental signal for drought stress or for cold stress leads to the accumulation of ABA which then triggers a common signal transduction pathway involved in the changes in gene expression common to both conditions. However, the signal transduction pathways involved in the induction of these genes in response to ABA, cold, and drought appears to be more complicated.

Recent experiments involving the temporal expression of one particular copy of a cold-regulated gene (*cor78*) in response to environmental signals other than low temperatures have suggested this gene responds to drought stress in a manner that is not observed during cold stress (Kazuko Y. and Kazuko S., 1992). Drought stress induces this gene very rapidly, (within 20 minutes) but accumulation of the message drops off after 40 minutes and remains at near control levels up to 3 hours after the initiation of the drought stress, whereupon it again begins to accumulate to significant levels. The transcript begins to accumulate in response to cold between 60 and 90 minutes after the plant has reached inducing temperatures and continues to accumulate steadily for 7 to

10 hours after which the message level appears to remain constant for as long as the plant is left in the cold (Yamaguchi-Shinozaki and Shinozaki, 1992). ABA treatment induces this gene with kinetics very similar to cold, with the mRNA accumulating within 60-90 minutes after treatment. These data suggest that this *cor78* responds differently to drought than it does to either cold or ABA, and that there are at least two different pathways that are capable of inducing it. Interestingly though, a second closely linked copy of *cor78* responds to drought, cold, and ABA with nearly identical kinetics (Yamaguchi-Shinozaki and Shinozaki, 1992).

A number of other studies have focused on the expression and regulation of several *cor* genes in *Arabidopsis* plants with altered ABA responses. In these studies, *Arabidopsis* plants with a mutation that renders them insensitive to exogenous applications of ABA (designated *abi1* for ABA insensitive 1), were either cold treated, drought stressed, or sprayed with ABA, and the induction of the *cor* genes was monitored. The results showed that the *cor* genes were still capable of responding to drought and cold stress, but were not induced by exogenous application of ABA (Nordin *et al.*, 1991; Gilmour and Thomashow, 1991). Since initial analysis of the *abi1* mutation has shown that the exogenously applied ABA does accumulate in such plants (suggesting that these plants are not deficient in ABA uptake), these studies appear to indicate that the induction of the *cor* genes in response to both cold and drought is not dependent on the plant's ability to perceive increases in internal ABA levels (Finkelstein and Sommerville, 1990). Such results point to the possibility that the ABA response operates via a different (or partially different) signal transduction pathway than does the drought or cold responses. It is noted however, that since the nature of the *abi1* mutation is not known, it is possible that endogenous ABA may still be functioning as an intermediate, and that some part of the pathway involved with the transmission of signal from exogenously applied ABA may be interrupted in the *abi1* mutants.

Finally, it has been demonstrated recently that there are at least two copies of one of the cold-regulated genes in *Arabidopsis* (designated *Kin1* and *Kin2*, and analogous to *cor6.6*). Both genes are induced by cold and by ABA, but *Kin1* is only induced by drought stress (Kurkela and Borg-Franck, 1992). This suggests that both the cold and the ABA response in the induction of this gene may be separable from the drought response. This also suggests that the signal transduction pathway involved in the drought response does not operate through ABA (at least for *Kin1*). Work on two distinct copies of the *cor15* gene from *Arabidopsis* appears to give similar results (Wilhelm, manuscript in preparation).

For future manipulation of the expression of the *cor* genes it is of interest to study how plants sense cold and the signal transduction pathway(s) that may be involved in altering gene expression in response to particular environmental stimuli. Traditionally, studies of this sort have relied upon the isolation and characterization genes that are regulated by the environmental stimulus, and the *cis* and *trans* acting factors controlling them. A considerable amount of success has been obtained in animal systems using this approach. The signal transduction pathway of post-transcriptionally regulated genes such as *c-fos* (Shyru *et al.*, 1989) and the iron transferrin genes (Leibold and Munro, 1988) as well as many transcriptionally regulated genes (Gronemeyer, 1992; Hunter and Karin, 1992; Karin and Smeal, 1992;) have been, or are being characterized through studies facilitated by the identification of key *cis* and *trans* acting factors from genes responsive to these stimuli.

III. POST-TRANSCRIPTIONAL REGULATION

Factors which bind specific sequences in RNA and alter its stability have been found in animals (Koeller *et al., 1989*; Ross, 1989), and are likely to be found in plants (Elliott *et al.*, 1989). There are two fairly well characterized systems involving changes in RNA stability in animal cells that have been studied. These are the regulation of *c-myc* and iron transferrin mRNA stability. In both systems, specific sequences in the 3' untranslated region (UTR) of the gene have been shown to be necessary for appropriate regulation. In the case of *c-myc* mRNA, the sequences of interest are a series of AUUUA repeats in it's 3' UTR. Repeats of this sequence when placed in the 3'UTR of a reporter gene can dramatically reduce the stability of its RNA (Shaw and Kamen, 1986). Specific proteins have been shown to bind to this AUUUA motif *in vivo* (Myer *et al.*, 1992) and site specific changes in this sequence that prevents binding of the AUUUA binding factor also result in a more stable RNA (Malter, 1989). With the iron transferrin mRNA, the 3'UTR was shown to impart an iron dependent decrease in the stability of RNA from a reporter gene. A hypothetical hairpin loop structure within the 'U' rich region of the 3' UTR was shown to bind a protein factor, and specific changes in these sequences that disrupted the hairpin formation also prevented the protein binding and resulted in a more stable RNA (Mullner and Kuhn, 1988). In plants, the ferridoxin I gene has been shown to be primarily regulated at the level of RNA stability (Elliott *et al.*, 1989). In this system, the sequences that regulate stability appear to be located in the 5' UTR, and reporter genes engineered with the ferridoxin 5' UTR become less stable in response to far red light. This system however, has not been as well characterized, and the action of any specific RNA binding factors has not been demonstrated. In the above cases, it is generally assumed that the binding of these specific sites in the 5' or 3' end of these messages protects these sites from endonucleases with specificities for the same or nearby sequences (Malter, 1989; Mullner *et al.*, 1989).

Other mechanisms which may be functioning to bring about changes in RNA stability are those which specifically alter the affinity of poly A binding proteins which are involved in protecting the 3' end of specific RNAs (Bernstein and Ross, 1989). It has been observed that generally mRNAs with longer poly A tails are usually more stable than mRNAs with shorter poly A tails (Shapiro *et al.*, 1987). The reason for this is thought to be due to the presence of poly A binding proteins (PABPs) that may be involved in protecting the 3' end of the RNA from 3'->5' exonucleases (Ross, 1989). There is some evidence to support the proposal that the AU rich regions which have been correlated with reduced RNA stability (including the AUUUA sequences in the *c-myc* gene) may compete for PABPs with the poly A tail, and thus leave the 3' end more open to degradation by exonucleases (Bernstein *et al.*, 1989).

Along with RNA stability, RNA splicing mechanisms offer points of control for gene regulation. It has been found that the presence of certain introns within a number of different structural genes can increase the expression level of these genes (Luehrsen and Walbot, 1991). The mechanisms of how the introns enhance the accumulation of these messages is not well understood. However, the data suggest that the introns, or the proteins acting on, or bound to the intron containing RNAs may protect these RNAs from nucleases present within the nucleus. This protection may be in the form of steric hindrance or competition for nuclease binding sites, and/or of increased efficiency of transport from the nucleus.

IV. TRANSCRIPTIONAL REGULATION

Although a number of post-transcriptionally regulated genes have been characterized, in most cases experiments have focused on genes that are regulated primarily at the transcriptional level and thus much has been learned about the functional components of the eucaryotic promoter. Most eucaryotic promoters have several common (but not ubiquitous) features that appear to be conserved. These are the TATA box, and upstream regulatory elements (UREs) (sometimes referred to as upstream activating sequences in yeast) (Dynan and Tjian, 1985)

The TATA box serves as the binding site for a general transcription factor (TFIID) (Greenblatt, 1991). Other general transcription factors necessary for basal level transcription appear to bind to or in someway interact with TFIID (Sawadogo and Sentenac, 1990; Sharp, 1992). Also, there appear to be factors that interact specifically with TFIID and facilitate the formation of the transcriptional initiation complex (Horikoshi *et al.*, 1988). The subsequent complex probably serves to recruit RNA polymerase II (Treisman and Maniatis, 1985). It is also thought that the TATA box complex is necessary for placement of the RNA polymerase in order to ensure that transcription is initiated at the appropriate site (Greenblatt, 1991). However, there are a number of promoters that do not appear to contain a functional TATA box, although they still support active transcription with a defined transcriptional initiation site (Pugh and Tjian, 1991). In these cases, however, it has been demonstrated that specific sequences around the start of transcription are required for appropriate initiation (Roy *et al.*, 1991).

UREs are short sequences in the promoter that act as binding sites for proteins that can interact either directly or indirectly with TFIID or other proteins in the transcription complex in order to facilitate complex formation and/or RNA polymerase II binding (Klein-Hitpass *et al.*, 1990). UREs can affect transcription even when they are positioned some distance from the transcription start site (up to several thousand bases away) (Struhl, 1987). However, it has been found that when such sequences were placed at varying distances from the start site, full activity was only observed when the distance to the TATA box was altered in multiples of ten to eleven bases (Takahashi *et al.*, 1986). However, as the distance from the start site was increased, the requirement for this periodicity for full activity was relaxed. This suggests that the URE binding proteins must be present in a specific orientation for enhanced RNA polymerase activity, and that this requirement may be fulfilled if there is sufficient distance between the URE and the site of complex formation. A likely model for how these URE binding factors work at variable distances from the transcription start is that they interact with or recruit other components of the transcription complex by a looping out of the intervening DNA. Therefore, if the distance between the site of URE factor binding and the assembly of the basal transcription complex is sufficient to allow enough flexibility in the DNA for the URE binding protein to be appropriately presented, then activation is possible. In fact, *in vitro* studies have shown, that if the concentration of bound and active URE binding proteins is high enough, full activation of a promoter may be achieved *in vitro*, even if the URE is present on a completely separate piece but linked piece of DNA (Muller *et al.*, 1989).

Much work has been focused on determining the exact mode of action of the *trans* acting factors that bind to the UREs and how they bring about enhanced transcription. There appear to be several classes of such factors with differing modes of action. One common class contains a characteristic acid activating domain, whose role was discovered by creating fusion proteins containing various portions of a known activating factor connected to a DNA binding domain specific for a sequence engineered into a reporter gene. What this acid activating domain specifically interacts with, and how it brings about increased transcription has not yet been elucidated. Early studies have indicated that such domains may interact directly with TFIID or with polymerase directly (Ingles *et al.*, 1991; Cullen, 1990). Alternatively, they may be involved in specifically interacting with nucleosomes and thus open regions of the promoter for TFIID binding (Taylor *et al.*, 1991). However, there are also indications that there may be other factors which are required for such acidic domains to be functional, and that these factors may act as adapters between the URE binding proteins and other components of the basal transcriptional complex (specifically TFIIA) (Wang *et al.*, 1992).

Another interesting and little understood aspect of URE binding factors is how such proteins recognize their appropriate binding sites. Since many of these factors recognize and bind to fairly short DNA sequences (4-10 bps), it is not clear what prevents them from binding inappropriately to similar sequences that are likely to be present throughout the genome. Currently, there are several common DNA binding motifs in URE binding factors that have been characterized. These include zinc fingers, helix turn helix, leucine zippers, and *c-myb* like binding factors. Although the gross interaction of the amino acids within these domains and the DNA itself are reasonably well understood (Johnson and McKnight, 1989), there are a number of interesting ambiguities concerning the specificity of the binding factors and the recognition of their appropriate binding sites. There are a number of cases in which the site to which a given factor binds is significantly degenerate (Payvar *et al.*, 1983; deVargas *et al.*, 1988; Weintraub *et al.*, 1991). How these factors recognize such different sequences, and what prevents them from binding inappropriately to other similar sequences found randomly in other promoters throughout the genome is not well understood (Johnson and McKnight, 1989). Also, there are a number of cases where there appears to be a family of very similar but distinct factors which all recognize the same sequence, but which appear to regulate different genes in different ways (Weintraub *et al.*, 1991).

In their review, Johnson and McKnight used the OCT family of transcription factors to serve as an illustration of how this type of system may operate. In this case, there are at least two distinct factors which both recognize the sequence TAATGARAT albeit with slightly different affinities, and which demonstrate virtually identical gene activating properties *in vitro*. OCT1 is ubiquitous in mammalian cells, but OCT2 is present only in lymphoid cells and plays a role in transcription from the Ig heavy chain promoter. Since OCT1 is ubiquitous, and binds to the same sequence, there is question as to why the Ig heavy chain promoter is not activated in non-lymphoid cells. Assuming the Ig heavy chain promoter is open, there appears to be nothing that prevents the binding and subsequent activation by OCT1. Although there are no significant differences in the DNA binding capacities of these two factors, there do appear to be some differences in their ability to interact with other proteins. For instance, OCT1 can interact with and form part of the ternary complex with the herpes virus activator protein, VP16, whereas OCT2 cannot. Also, OCT2 has a leucine zipper-like motif outside its DNA binding domain that OCT1 does not have. It has been suggested that this zipper domain may allow the OCT2 protein to interact with factors that do not recognize OCT1, and that these separate protein:protein interactions are responsible for the binding and/or activating differences observed *in vivo*.

Another family of seemingly promiscuous binding factors may be particularly relevant to the study of our cold-regulated genes. Of interest is a family of leucine zipper type binding factors called GBFs (G-Box binding factors), which recognize variations of the core sequence CACGTG (G-Box) (Schindler *et al.*, 1992; Williams *et al.*, 1992). These G-Box sequences were first characterized as protein binding sites that played some role in the expression of light regulated genes such as the small subunit of Rubisco (Guiltinan *et al.*, 1988). Also, very similar sequences were found to be important (if not sufficient) for the expression of a number of ABA-regulated genes (Guiltinan *et al.*, 1990; Mundy *et al.*, 1990). Subsequently, similar sequences have been found in the promoters of a number of very differently regulated genes from a large number of different species. As examples, in *A. thaliana* alone, the *HSP70-1, adh, chs, rbcS-1A,* and all of the known *cor* genes (which have also been shown to be ABA-regulated) contain similar sequences in their promoters (Williams *et al.*, 1992). Whether the binding of factors to these sites is sufficient for the induction of any of these genes, or if binding simply functions to enhance or allow the transcription of already activated genes is not known. There is, however, some evidence that points to the former. It has been shown that a tetramer of a 21 bp piece of DNA containing the sequence GGACGCGTGGC was able to confer ABA and desiccation induced regulation to the both the -46 and -90 minimal CaMV promoter fragments (Lam and Chua, 1991). The sequence within this tetramer is very similar to the sequence GGACACGTGGC which has been shown to bind a similar factor and has been shown to play an important role in the induction of Em1, an ABA-regulated gene from wheat (Guiltinan *et al.*, 1990). It should be noted, however, that a tetramer of the sequence TACGTGGC, which is implicated in ABA regulation from several rice and cotton genes, was incapable of imparting ABA regulation in tobacco, even when specific binding of the endogenous GBF (in this case the GBF homologue from tobacco named TAF1) was demonstrated (Oeda *et al.*, 1991).

Much recent work has been directed at determining how these G-box like sequences can control such a diverse set of genes. In *Arabidopsis*, at least three distinct binding factors which recognize G-Box sequences have been cloned and characterized (Schindler et al., 1992). At least two such binding activities have also been isolated from nuclear extracts of leaves from parsley, cauliflower, wheat, rice, and tobacco (Armstrong *et al.*, 1992; Williams *et al.*, 1992; Guiltinan *et al.*, 1990; Mundy *et al.*, 1990; Katagiri *et al., 1989*), and the genes for a number of these binding factors have also been cloned (Armstrong *et al.*, 1992; Guiltinan *et al.*, 1990; Oeda *et al.*, 1991). As was the case for OCT1 and OCT2, it appears that these binding factors are able to differentiate between several genes with G-box binding sites within the same nucleus (Williams *et al.*, 1992).

The fact that there appear to be several differentially regulated genes all bound by GBFs within a given cell suggests a number of possible explanations for how this family of binding factors operate. It is possible that the promoters for these different genes are bound by other factors which prevent binding of the GBFs when the genes are inactive. In this scenario, when the appropriate environmental signal is perceived, these blocking factors are moved away, and the promoters become "open" for G-Box binding and subsequent gene activation. Another possibility is that there are a large number of GBFs that are all "activated" by specific environmental signals and that all recognize subtly different flanking sequences around their respective G-Boxes. In this case, when the appropriate environmental signal is perceived and the GBF is activated, it then binds only to the promoters that contain G-Boxes in the context of specific flanking sequences. Alternatively, there may only be a limited number of GBFs, but each type could be specifically altered in different ways by various environmental signals. Such alterations could cause the factors to recognize subtle differences in the G-Box sequences or in the various other proteins that are likely to be present on the promoters of their respective genes. Such alterations may be in the form of specific phosphorylations, acetylations, or methylations, or they may be the result of other protein:protein interactions (as was suggested for OCT1 and OCT2 specificities). Finally, the reality of how these factors bring about the specific regulation of the many different genes may well be the result of a combination or subtle derivation of any of the possibilities just discussed.

A number of significant observations have been made in investigating how the GBFs operate, which have lent support to the various models just discussed. These are: 1) G-box binding factors, like other known luecine zipper DNA binding factors (see Ziff, 1990 for a review), are capable of forming heterodimers (Schindler *et al.*, 1992). This means that with a limited number of GBF monomers many novel GBF dimers could be formed. If it is shown that different GBF combinations have slightly different binding specificities and sensitivities to different environmental signals, then the specificity requirement will be met. 2) GBFs can be modified via phosphorylation, and this phosphorylation affects the GBFs' gene activating abilities (Klimczak *et al.*, 1992). It will be interesting to see if any specific modifications occur in response to a particular environmental signal, and if so, whether any of these modifications alter the ability of the GBF to bind subtly different G-Box sequences or form specific GBF heterodimers. 3) *In vitro* binding studies with several different GBFs isolated from a single nuclear preparation have demonstrated distinct binding activities in a sequence dependent manner (Williams *et al.*, 1992; Schindler *et al.*, 1992). Also, GBFs from parsley interact with other nuclear factors and that these interactions alter their binding specificities (Armstrong *et al.*, 1992). Thus, it appears that given GBFs can be distinguished by their ability to recognize and bind subtly different G-Box sequences. If it is demonstrated that there are indeed many different GBFs (or heterodimer combinations), then again the specificity requirements will be met. 4) *Arabidopsis* GBF3 and tobacco TAF1 both appear to be expressed primarily in the roots, while *Arabidopsis* GBF1 and GBF2 are expressed primarily in green tissue (Schindler *et al*, 1992; Odea *et al.*, 1991). This means that within the GBF gene family, certain members can be expressed in a tissue specific manner. Therefore, at least a portion of the specificity requirements may be met by limiting the expression of certain GBF forms to specific tissues. Expanding on this idea, it is also possible that the presence of other additional factors might inhibit the expression of genes that would otherwise be activated by GBFs in specific tissues (i.e. tissue specific silencers).

Finding and characterizing UREs and the proteins which bind them is only the initial step in elucidating any given signal transduction pathway. One must next determine the factors which influence the presence of, the activation of, and the binding of these proteins to the promoters. Such factors include the spatial and temporal regulation of the expression of the genes which encode these DNA binding proteins, and also the proteins involved in the phosphorylation or other post-translational modifications of these proteins which may alter their ability to bind to their specific sequences or alter their ability to interact appropriately with the transcriptional apparatus. For example, the chromatin structure in and around the promoter has been demonstrated to alter the binding pattern of the essential components of the transcriptional apparatus (Laybourn and Kadonaga (1991); see Eissenberg and Elgin, (1991) for a review). Finally, the spatial orientation and packing of the chromatin within the nucleus and the channels through it may even play a role by determining which mRNAs are allowed to leave the nucleus and which are physically blocked by the maze of proteins and DNA (Blobel, 1985; Chang and Sharp 1990).

Much general knowledge has been learned about how eucaryotic genes may be regulated. However, at the time this dissertation was begun, little was known about how plants perceived environmental signals or the signal transduction pathways that existed to bring about alter gene expression in response to changes in the environment. There has also been much learned concerning the changes in physiology and biochemistry that occur in plants that are placed at low temperature. However, few conclusive studies have been done to illuminate the affects of any of the given changes that are known to occur. In order to learn more about both environmental signal perception, and to obtain the tools to study the molecular aspects of cold stress physiology, the work in this dissertation was undertaken. Specifically, the goal of this work was to clone cold-regulated genes from *Arabidopsis*, and to study the regulation of those genes to gain insight into how *Arabidopsis* senses low temperatures and brings about changes in gene expression.

Literature Cited

Armstrong GA, Weisshaar B, Hahlbrock (1992) Homodimeric and heterodimeric leucine zipper proteins and nuclear factors from Parsley recognize diverse promoter elements with ACGT cores. The Plant Cell 4, 525-537

Berstein P, Ross J (1989) Poly(A)-poly(A) binding protein and the regulation of mRNA stability. TIBS 14, 373-377

Bernstein P, Peltz S, Ross J (1989) The poly(A)-poly(A) binding protein complex is a major determinant of mRNA stability *in vitro*. Mol. Cell. Biol. 9, 659-670

Blobel G (1985) Gene gating: A hypothesis. PNAS 83, 8527-8529

Brandts EJ (1967) Thermobiology. Academic Press, New York, pp25-73

Carpenter JF, Crowe JH (1988) The mechanism of cryoprotection of proteins by solutes. Cryobiology 25, 244-255

Chang DD, Sharp PA (1990) Messenger RNA transport and HIV *rev* regulation. Science 249, 614-615

Chen THH, Gusta LV (1983) Abscisic acid-induced freezing resistance in cultured plant cells. Plant Physiol. 73, 71-75

Chen HH, Li PH, Brenner ML (1983) Involvement of abscisic acid in potato cold acclimation. Plant Physiol. 71, 362-365

Cullen BR (1990) The HIV-1 Tat protein: An RNA sequence specific processivity factor. Cell 63, 655-657

Daie J, Campbell WF (1981) Response of tomato plants to stressful temperatures. Plant Physiol. 67, 26-69

DeVargas L, Pargellis C, Hasan N, Bushman E, Landy A (1988) Autonomous DNA binding domains of integrase recognize two different sequence families. Cell 54, 923-929

Dynan W, Tjian R (1985) Control of eukaryotic messenger RNA synthesis by sequence specific DNA binding proteins. Nature 316, 774-777

Eissenberg JC, Elgin SCR (1991) Boundary functions in the control of gene expression. Trends in Genetics 7, 335-340

Elliott R, Dickey L, White M, Thompson W (1989) *Cis*-acting elements for light regulation of pea ferredoxin I gene expression are located within the transcribed sequences. The Plant Cell 1, 691-698

Finkelstein RR, Sommerville CR (1990) Three classes of abscisic acid (ABA)-insensitive mutations of *Arabidopsis* define genes that control overlapping subsets of ABA responses. Plant Physiol. 94, 1172-1179

Gatenby AA (1992) Protein folding and chaperonins. Plant Mol. Biol. 19, 677-687

Gilmour SJ, Hejela RK, Thomashow MF (1988) Cold acclimation in *Arabidopsis thaliana*. Plant Physiol. 87, 745-750

Gilmour SJ, Thomashow MF (1991) Cold acclimation and cold-regulated gene expression in ABA mutants of *Arabidopsis thaliana*. Plant Mol. Biol. 17, 1233-1240

Gordon-Kamm WJ, Steponkus P (1984) The influence of cold acclimation on the behavior of the plasma membrane following osmotic contraction of isolated protoplasts. Protoplasms 123, 161-173

Gordan-Kamm WJ, Steponkus P (1984) The behavior of the plasma membrane following osmotic contraction of isolated protoplasts: Implications in freezing injury. Protoplasma 123, 83-94

Greenblatt J (1991) Roles of TFIID in transcription initiation by polymerase II. Cell 66, 1067-1070

Gronemyer H (1992) Control of transcriptional activation by steroid hormone receptors. The FASEB Journal 6, 2524-2529

Guiltinan MJ, Marcotte WR, Quatrano RS (1990) A plant leucine zipper protein that recognizes an abscisic acid response element. Science 250, 267-270

Guy C (1990) Cold acclimation and freezing stress tolerance: role of protein metabolism. Ann. Rev. of Plant Physiol. 41, 187-223

Hajela R, Horvath DP, Gilmour SJ, Thomashow MF (1990) Molecular cloning and expression of *cor* (cold-regulated) genes in *Arabidopsis thaliana.* Plant Physiol. 93, 1246-1252

Horikoshi M, Hai T, Lin Y, Green M, Roeder R (1988) Transcription factor ATF interacts with TATA factor to facilitate establishment of a preinitiation complex. Cell 54, 1033-1042

Hunter T, Karin M (1992) The regulation of transcription by phoshorylation. Cell 70, 375-387

Inges C, Shales W, Cress S, Treizenberg S, Greenblatt J (1991) Reduced binding of TFIID to transcriptionally compromised mutants of VP16. Nature 351, 588-590

Johnson P, McKnight S (1989) Eucaryotic transcriptional regulatory proteins. Ann. Rev. Biochem. 58, 799-839

Karin M, Smeal T (1992) Control of transcription factors by signal transduction pathways: the beginning of the end. TIBS 17, 418-422

Yamaguchi-Shinozaki K. and Shinozaki K. (1992) Characterization of the expression of a desiccation-responsive rd29 gene of *Arabidopsis thaliana* and analysis of its promoter in transgenic plants. Mol. Gen. Genet. in press.

Klein-Hitpass L, Tsai S, Weigel N, Allan G, Riley D, Rodriguez R, Schrader W, Tsai M, O'Malley B (1990) The progesterone receptor stimulates cell-free transcription by enhancing the formation of a stable preinitiation complex. Cell 60, 247-257

Klimczak LJ, Schindler U, Cashmore T (1992) DNA binding activity of the *Arabidopsis* G-box binding factor GBF1 is stimulated by phosphorylation by casein kinase II from broccoli. The Plant Cell 4, 87-98

Koeller D, Casey J, Hentze M, Gerhardt E, Lee-Nien C, Klausner R, Harford J (1989) A cytosolic protein binds to structural elements within the iron regulatory region of the transferrin receptor mRNA. PNAS 86, 3574-3578

Kurkela S, Borg-Franck M (1992) Structure and expression of *kin2*, one of two cold- and ABA-induced genes of *Arabidopsis thaliana.* Plant Mol. Biol. 19, 689-692

Lam E, Chua N-H (1991) Tetramer of a 21-base pair synthetic element confers seed expression and transcriptional enhancement in response to water stress and abscisic acid. J. Biol. Chem. 266, 17131-17135

Lang V, Heino P, Palva ET (1989) Low temperature acclimation and treatment with exogenous abscisic acid induce common polypeptides in *Arabidopsis thaliana* (L.) Heynh. Theoretical and Applied Genetics 77, 729-734

Laybourn JP, Kadonaga JT (1991) Role of Nucleosomal cores and histone H1 in regulation of transcription by RNA polymerase II. Science 254 238,244

Levitt J (1980) Responses of plants to environmental stress. Chilling, freezing, and high temperature stresses. Ed 2, Academic Press, New York

Liebold EA, Munro HN (1988) Cytoplasmic protein binds to a highly conserved sequence in the 5' untranslated region of ferritin heavy- and light-subunit mRNAs. PNAS 85, 2171-2175

Luehrsen K, Walbot V (1991) Intron enhancement of gene expression and the splicing efficiency of introns in maize cells. Mol. Gen. Genet. 255, 81-93

Malter J (1989) Identification of an AUUUA-specific messenger RNA binding protein. Science 246, 664-666

Meyerowitz EM (1989) *Arabidopsis*, a useful weed. Cell 56, 263-269

Mohapatra SS, Poole RJ, Dhindsa RS (1988) Abscisic acid regulated gene expression in relation to freezing tolerance in alfalfa. Plant Physiol. 87, 468-473

Muller H, Sogo J, Schaffner W (1989) An enhancer stimulates transcription in *trans* when attached to the promoter via a protein bridge. Cell 58, 767-777

Mullner E, Kuhn L (1988) A stem loop in the 3' untranslated region mediates iron dependent regulation of the transferrin receptor mRNA stability in the cytoplasm. Cell 53, 815-825

Mullner E, Neupert B, Kuhn L (1989) A specific mRNA binding factor regulates the iron-dependent stability of cytoplasmic transferrin mRNA. Cell 58, 373-382

Mundy J, Yamaguchi-Shinozaki K, Chua N-H (1990) Nuclear proteins bind conserved elements in abscisic acid-responsive promoter of a rice *rab* gene. PNAS 87, 1406-1410

Myer V, Lee S, Steitz J (1992) Viral small nuclear ribonuclearproteins bind a protein implicated in messenger RNA destabilization. PNAS 89, 1296-1300

Nordin K, Pekka H, Palva E (1991) Separate signal pathways regulate the expression of a low-temperature-induced gene in *Arabidopsis thaliana* (L.) Heynh. Plant Mol. Biol. 16, 1061-1071

Odea K, Salinas J, Chua N-H (1991) A tobacco bZip transcription activator (TAF-1) binds to a G-box-like motif conserved in plant genes. The EMBO Journal 10, 1793-1802

Payvar F, DeFranco D, Firestone G, Edgar B, Wrang O (1983) Sequence-specific binding of glucocorticoid receptor to MTV DNA at sites within and upstream of the transcribed region. Cell 35, 381-392

Pugh BF, Tjian R (1991) Transcription from a TATA-less promoter requires a multisubunit TFIID complex. Genes and Dev. 5, 1935-1945

Ross J (1989) The turnover of messenger RNA. Scientific American Apr. pp48-55

Roy A, Meisterernst M, Pogmonec P, Roeder R (1991) Cooperative interaction of an initiator-binding transcription initiation factor and the helix-loop-helix activator USF. Nature 354, 245-248

Sakai A, Larcher W. (1987) Frost survival of plants; response and adaptation to freezing stress. Spring-Verlag, New York

Sawadogo M, Setenac A (1990) RNA polymerase B(II) and general transcription factors. Ann. Rev. Biochem. 59, 711-754

Schindler U, Terzaghi W, Beckmann H, Kadesch T, Cashmore T (1992) DNA binding site preferences and transcriptional activation properties of the *Arabidopsis* transcription factor GBF1. The EMBO Journal 11, 1275-1289

Schindler U, Menkens AE, Beckmann H, Ecker JR, Cashmore T (1992) Heterodimerization between light-regulated and ubiquitously expressed *Arabidopsis* GBF bZip proteins. The EMBO Journal 11, 1261-1273

Shapiro D, Blume J, Nielson D (1987) Regulation of messenger RNA stability in eucaryotic cells. Bio. Essays 6, 221-226

Sharp P (1992) TATA-binding protein is a classless factor. Cell 68, 819-821

Shaw G, Kamen R (1986) A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell 46, 659-667

Shyru A-B, Greenberg M, Belasco G (1989) The *c-fos* transcript is targeted for rapid decay by two distinct mRNA degradation pathways. Genes and Dev. 3, 60-72

Steponkus PL (1984) Role of the plasma membrane in freezing injury and cold acclimation. Annu. Rev. Plant Physiol. 35, 543-585

Steponkus P, Lynch D (1989) Freeze/thaw induced destabilization of the plasma membrane and the effects of cold acclimation. J. Bioenerg. Biomembr. 21, 21-41

Struhl K, (1997) Promoters, activators proteins, and the mechanisms of transcriptional initiation in yeast. Cell 49, 295-297

Takahashi K, Vigneron M, Matthes H, Wildman A, Zenke M, Chambon P (1989) Requirement of stereospecific alignments for initiation from simian virus 40 early promoter. Nature 319, 121-126

Taylor I, Workman J, Schuetz T, Kingston R (1991) Facilitated binding of GAL4 and heat shock factor to nucleosomal templates: Differential function of DNA-binding-domains. Genes and Dev. 5, 1285-1298

Thomashow M. (1990) Molecular genetics of cold acclimation in higher plants. In Scandalios JG, and Wright TRF, eds. Advances in genetics, Vol. 28, Genetic Responses to Environmental Stress. Academic Press, New York

Thomashow M, Gilmour S, Hajela R, Horvath D, Lin C, Guo W (1990) Studies on cold acclimation in *Arabidopsis thaliana.* IN, AB Bennett, SD O'Neill, eds, Horticultural Biotechnology. Wiley-Liss, New York, pp305-314

Treisman R, Maniatis T (1985) Simian virus40 enhancer increases the number of RNA polymerase II molecules on linked DNA. Nature 315, 72-75

Tseng M-J, Li PH (1990) Alterations of gene expression in potato (*Solanum commersonii*) during cold acclimation. Plant Physiol. 78, 538-547

Wang W, Gralla J, Carey M (1992) The acidic activator GAL4-AH can stimulate polymerase II transcription by promoting assembly of a closed complex requiring TFIID and TFIIA. Genes and Dev. 6, 1716-1727

Weintraub H, Davis R, Tapscott S, Thaymer M, Kraus M, Benezera R, Blackman T, Turner D, Rupp R, Hollenberg S, Zhuang Y, Lassar A (1991) The *myoD* gene family: Nodal point during specification of the muscle cell lineage. Science 251, 761-766

Weiser CJ (1970) Cold resistance and injury in woody plants. Science 169, 1269-1278

Williams ME, Foster R, Chua N-H (1992) Sequences flanking the hexameric G-box core CACGTG affect the specificity of protein binding. The Plant Cell 4, 485-496

Chapter 2

CLONING AND EXPRESSION OF COLD-REGULATED GENES FROM *ARABIDOPSIS THALIANA*

SUMMARY

Previous work has demonstrated specific changes in the population of mRNAs in *Arabidopsis thaliana* during incubation at 5^oC (a treatment known to acclimate plants to low temperatures and increase freezing resistance). Consequently, four clones (pHH28, pHH29, pHH67, and pHH7.2) were isolated from a cDNA library made from mRNA of cold-treated *Arabidopsis*. These clones represented distinct mRNAs (of sizes 2.5, 0.55, 0.65, and 1.1 kb) which specifically accumulated 10-30 fold during a three day cold treatment. These cold-regulated (*cor*) mRNAs also accumulate in response to drought stress. They do not however, accumulate in response to heat shock. The *cor* messages accumulate in plants placed at temperatures of about 10^o-12^oC for three days. Nuclear runon experiments suggests that three of these mRNAs (represented by clones pHH28, pHH29, and pHH7.2) are primarily post-transcriptionally regulated. The mRNA represented by clone pHH67 appears to be primarily transcriptionally regulated. The implications for signal transduction concerning these results are discussed.

INTRODUCTION

Many plant species have the ability to "cold harden" or acclimate to freezing temperatures when first exposed to low, non-freezing temperatures. Such cold acclimated plants become increasingly freezing tolerant and are better able to survive frost (Levitt, 1980; Sakai and Larcher, 1987). In addition to this cold acclimation process (Graham and Patterson, 1982), ABA application at ambient temperatures has been shown to bring about increased freezing tolerance in intact plants as well as in cell and tissue cultures (Rikin *et al.*, 1975; Chen *et al.*, 1979; Chen and Gusta, 1983; Keith and McKersie, 1986; Orr *et al.*, 1986; Reaney and Gusta, 1987).

Several physiological and biochemical changes are associated with cold acclimation. Changes in membrane composition and organization, such as increased membrane fluidity and desaturation of membrane fatty acids are believed to play important roles in maintaining cellular integrity (Sakai and Larcher, 1987). Other cellular changes like alterations in levels of free sugars, amino acids and soluble proteins have been reported to be associated with and to have important roles in cold acclimation in general and freezing tolerance in particular (Levitt, 1980). However, the exact roles of these changes during cold acclimation are not yet fully understood. Some of the sugars, amino acids and proteins that accumulate during cold acclimation may physically help prevent freezing, but no direct cause and effect relationship has been clearly established.

Endogenous levels of ABA have been shown to increase during cold acclimation and prior to development of freezing tolerance (Kasperska-Palacz *et al.*, 1977; Daie and Campbell, 1981; Chen *et al*, 1983). It has even suggested that cold induces an increase in endogenous ABA levels which then acts as the stimulus for development of cold hardiness, a hypothesis which remains to be tested directly (Chen *et al.*, 1983).

Cold hardiness is a heritable trait (see Thomashow, 1989 for a review). Many investigators have observed accumulation of new mRNAs and novel polypeptides during cold acclimation and prior to development of freezing tolerance (Graham and Patterson, 1982; Cloutier, 1983; Guy and Haskell 1989; Kurkela *et al.*, 1988; Gilmour *et al.*, 1988). These studies have relied both on *in vitro* translation of isolated messages as well as *in vivo* labelling of polypeptides. Also, cytoplasmic protein synthesis inhibitors like cycloheximide have been observed to block cold acclimation (Chen *et al*, 1983; Trunova, 1982). However, chloramphenicol, a chloroplast protein synthesis inhibitor has no effect on cold acclimation. All of these observations support the earlier suggestions that regulation of nuclear gene activity may be involved in cold acclimation (Weiser, 1970). However, the specific genes, and the mechanisms and levels of regulation of action remain unknown. To establish clearly a relationship between gene expression and the increase in freezing tolerance, the genes in question must be isolated, identified, and characterized.

It has previously been established (Kurkela *et al.*, 1988; Gilmour *et al.*, 1988) that *Arabidopsis thaliana*, like many other herbaceous plants, can cold acclimate. I decided to use *Arabidopsis* for further investigations on the molecular biology of cold acclimation in plants. *Arabidopsis* is a small crucifer with a short life cycle (about six weeks), has a small genome of about 70 million bp (with very little repetitive DNA), and is amenable to gene transfer and regeneration of transgenic plants (Meyerowitz, 1989). The availability of many well characterized mutants further adds to its utility in molecular genetic studies. Finally, since *Arabidopsis* is a crucifer, what is learned from studies on it may be generally applicable to other, economically important crucifers such as rapeseed mustards, and *Brassica oleracea* (broccoli, cauliflower, cabbage and a host of others).

In this chapter, the isolation and molecular cloning of several cold-regulated (*cor*) genes from a cDNA library made from cold acclimated *Arabidopsis* is reported. Initial characterization of some of these cDNA clones is presented, and the environmental factors that may play a role in their regulation are explored. Finally, the level (transcriptional or post-transcriptional) of regulation of these *cor* genes is examined.

MATERIALS AND METHODS

Growth and treatment of plants

Plants and calli were grown as previously described (Gilmour *et al.*, 1988). Plant material was flash frozen in liquid nitrogen, pulverized to a fine powder and stored at -80°C until used. To study the effects of various temperatures or other stresses, plants grown under constant illumination at 23°C for 18 - 20 days were used.

To determine the minimum temperature at which the *cor* genes were induced, plants were moved to growth chambers pre-set to 5, 7, 10, 12, 15 or 17°C. For all other experiments plants were moved to growth chambers set at 5°C and incubated for the desired times. For time course of cold-induction studies, samples were withdrawn after 1 and 4 hours and at subsequent 4 hour intervals until 24 hours, then at 3, 7 and 14 days, frozen in liquid nitrogen and stored at -80°C. Similarly, for the time course of deacclimation, plants that had remained in cold for 3, 7, or 14 days were returned to growth chambers set at 23°C and samples removed after 1 and 4 hours and at subsequent 4 hourly intervals to 24 hours and then at 3, 7, and 14 days, frozen in liquid nitrogen and stored at -80°C. ABA treated plants were sprayed to runoff with a 100 uM solution of (+/-) ABA (mixed isomers obtained from Sigma Chem. Corp.). Pots were covered with plastic wrap and placed under continuous fluorescent light. Samples were taken at various times up to 24 hours. Heat stressed plants were moved to a chamber set at 37°C and incubated for 3 hours before harvesting and storage at -80°C. Drought stress was induced by withholding water for up to 7 days.

RNA extraction and northern analysis

Poly A mRNA from *Arabidopsis* was extracted as previously described (Gilmour *et al.*, 1988). Initial northern analyses were done on poly A^+ mRNA from ecotypes Columbia and Landsberg erecta to ascertain the universality of the induction of the selected messages. All later northern analyses used total RNA from Landsberg plants.

Preparation and screening of cDNA library

Double stranded cDNA used for making a library in EcoRI-cut lambda ZAP was synthesized essentially by the method of Gubler and Hoffman (1983) using AMV reverse transcriptase (Promega). Double stranded (DS) cDNAs were methylated with EcoRI methylase and ligated to EcoRI linkers (NEB). These were then cut with EcoRI, and fractionated on a Sepharose CL4-B column using established, published methods (Maniatis *et al.*, 1982). DS cDNA fractions 0.5 kb and larger were pooled, ligated to EcoRI cut, phosphotased lambda ZAP, and the recombinant phage packaged *in vitro* with Packagene (Promega) extract. The resulting phage particles were plated on *E. coli* BB4 cells supplied with lambda ZAP (Stratagene). At least 5×10^5 primary recombinant plaques were obtained, amplified one cycle and frozen for storage at -80°C in 7% DMSO (Maniatis *et al.*, 1982). All subsequent plating was done by scraping a small amount of phage from the frozen stock without any secondary amplification to prevent any possible loss of recombinants.

Differential screening was done on plaque lifts on Nytran (Schleicher & Schull) and at least half the library equivalent was screened in one experiment, using ^{32}P labeled cDNA probes made from control or cold acclimated plant or callus mRNA. Probed filters were sandwiched between Cronex lighting plus (DuPont) intensifying screens and Kodak AR5 X-ray film at -80°C. Selected plaques were purified, rescreened as mentioned in 'results' and recombinant cDNA 'excised' out of the phage in plasmid bluescript SK^- using the biological rescue recommended by Stratagene.

Genomic blots and Southern analysis of cDNAs

Southern analysis was performed on EcoRI cut recombinant plasmids to establish the relationships of the isolated clones. Southern analysis was also done on restricted genomic DNA from *Arabidopsis* plants to identify genomic segments and to determine the copy number of these genes. Northern analysis was performed after formaldehyde gel electrophoresis (Maniatis *et al.*, 1982) using poly A mRNA or total RNA isolated as above. Nytran (Schleicher & Schull) was used for Southern and northern blotting.

Nuclear isolation and runon transcription

Isolation of nuclei for runon transcription employed the method of Feinbaum (1987). Runon transcription using ^{32}P labelled UTP was done as described by Ausubel *et al.*, (1987), RNA was purified and used to probe slot blots of gel purified isolated inserts of DNA from selected recombinant plasmids.

RESULTS

Cloning of cold-regulated genes

Cold acclimation in *Arabidopsis* is accompanied by stable changes in the mRNA population as demonstrated by one or two dimensional gels of *in vitro* translation products (Gilmour *et al.*, 1988; Kurkela *et al.*, 1988). Differential screening of a lambda Zap/cDNA library made from cold acclimated *Arabidopsis* mRNA yielded about twenty cold-regulated cDNA clones. Southern hybridization indicated that four different genes (or gene families) were represented by these 20 clones. A representative clone for each gene (or gene family) was selected and used to probe northern blots of poly A^+ or total mRNA from control, cold acclimated and deacclimated plants of *A. thaliana* ecotype Landsberg (see Table 2.1). Four discrete mRNA species ranging in size from 0.5 to 2.4 kb were found to be cold-regulated (Figure 2.1). A non-cold-regulated gene represented by pHH25 was isolated to use as a control. It is evident from these experiments that the recombinants selected represent messages which are either absent or present at very low basal levels in both control and deacclimated plants but accumulate significantly during cold adaptation.

Cold-regulated genes are induced at temperatures between 10°C and 15°C

In order to determine the maximum temperature at which these four cold-regulated RNAs start to accumulate, plants were incubated for three days in growth chambers set at temperatures ranging from 17°C to 5°C. Northern blots of RNA isolated from these plants were probed using the inserts from selected cold-regulated cDNA clones. The results demonstrate that the threshold temperature for inducing the *cor* genes is reached at between 10 and 15°C (Figure 2.2).

Table 2.1

General Characteristics of cor cDNA Clones

Clone Designation	Insert Size	Transcript Size	Representation in Library[a]
	kbp	*kb*	%
pHH7.2	1.1	1.4	0.02
pHH28	1.3	2.5	0.03
pHH29	0.6	0.6	0.1
pHH67	0.7	0.7	0.1

[a] Inserts from the various *cor* cDNA clones were ^{32}P-labeled (10), hybridized with plaque lifts of the cDNA library, and the percentage of the plaques displaying hybridization estimated.

Figure 2.1 Northern analysis of *cor* gene transcripts. Total RNA (5 ug) isolated from *Arabidopsis* leaves and stems was fractionated on formaldehyde agarose gels, transferred to nytran, and hybridized with ^{32}P-labled cDNA inserts as indicated. N, Nonacclimated; A, acclimated at 5^{o}C for 3 days; D, deacclimated (3 days at 5^{o}C then 1 day at 22^{o}C). Transcript sizes are given in kb.

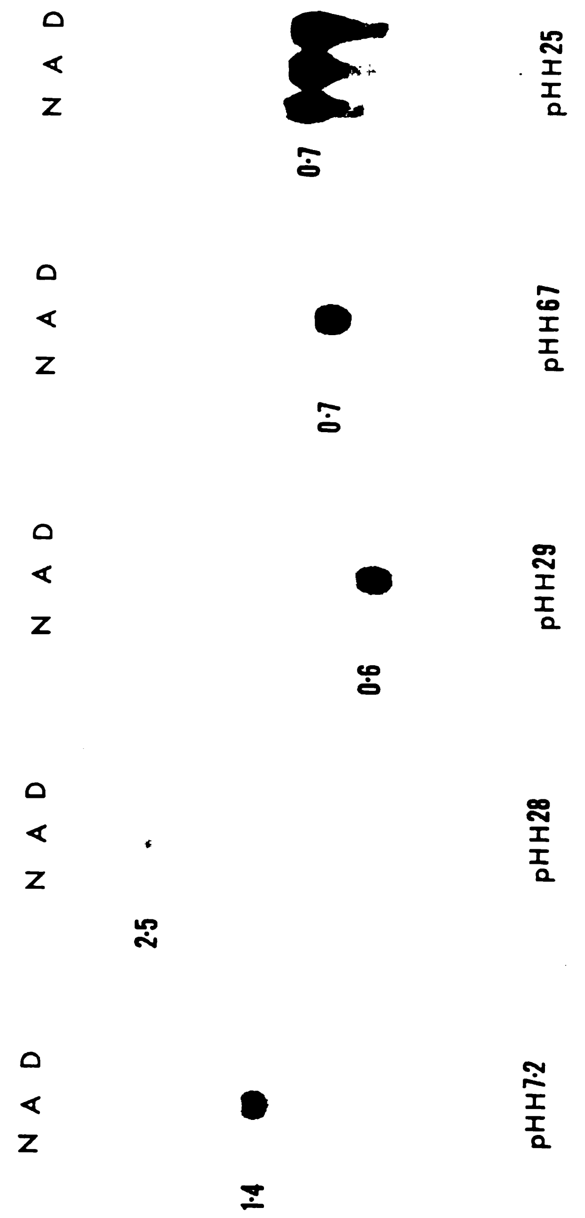

N A D
N A D
N A D
N A D
N A D
0·7
0·7
0·6
2·5
1·4
pHH25
pHH67
pHH29
pHH28
pHH7·2

Figure 2.1

Figure 2.2 Northern analysis of *cor* gene transcripts after plants were plants were placed at various temperatures (as indicated) for three days. 10 ug of total RNA was fractionated on formaldehyde agarose gels, blotted to Nytran, and hybridized to nick translated plasmids containing the indicated *cor* cDNAs.

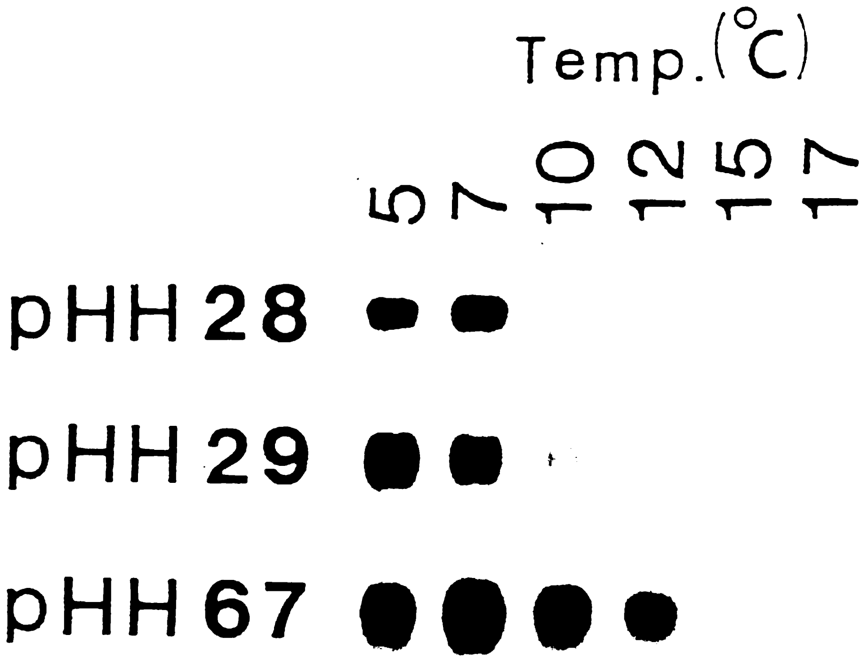


Figure 2.2

Cold-regulated genes are controlled both transcriptionally and post-transcriptionally

The level at which the selected cold-regulated (*cor*) genes are regulated was investigated using nuclear runon transcription assays. This study demonstrates that the gene represented by the cDNA clone pHH67 is transcribed at a very low level in control plants. Its transcription increases dramatically when the plant is placed at $5^{o}C$ for three days (Table 2.2). This result suggests a strong transcriptional component in its regulation. However, the increase in the rate of transcription is insufficient to explain the levels to which the RNAs corresponding to pHH28, pHH29, and pHH7.2 accumulate (Table 2.2). Also, it is interesting to note that even under control conditions, where the level of accumulation of these cold-regulated messages is nearly zero, the genes corresponding to pHH28, and pHH7.2 are transcribed at a rate at least as high as any of the other control genes in these experiments (Figure 2.3, and other data not shown). These data suggest that the genes represented by pHH28, pHH7.2, and pHH29 are regulated at a post-transcriptional level.

Cold-regulated genes are induced by drought stress, but not by heat shock

When plants are subjected to freezing temperatures, one major form of injury is directly related to cellular dehydration caused by extra-cellular ice formation (Steponkus, 1984), it was of interest to determine if drought stress would also induce any of these cold-regulated genes. To do this, water was withheld from two week old plants, their relative water content was determined, and their RNA was extracted. Northern analysis demonstrated that drought stress is also capable of inducing all four *cor* genes (Figure 2.4).

Table 2.2

Relative Transcription Rates and Transcript Levels of cor Genes in Cold Acclimated Versus Nonacclimated Plants

Parameter	**cDNA Clone**				
	pHH7.2	**pHH28**	**pHH29**	**pHH67**	**pHH25**
			-fold increase in cold-acclimated plants		
Transcription rate[a]	1.2 ± 0.2	1.8 ± 0.3	2.4 ± 1.1	9.6 ± 2.3	1.7 ± 0.3
Transcript level[b]	8.6 ± 1.9	22 ± 5.0	11 ± 4.3	26 ± 8.4	0.8 ± 0.03

[a] Three independent sets of nuclei were isolated from control and 3 d cold-acclimated plants and the relative transcription rates were determined by nuclear run-on transcription assays as described in "Materials and Methods." Values are the mean (± SE) fold increase in transcription rate in cold-acclimated plants. [b] Total RNA was isolated from control and cold-acclimated plants (either leaves and stems or whole plants) and the steady state transcript levels of the indicated genes were determined by Northern analysis as described in "Materials and Methods." Values are the mean (± SE) fold increase in transcript level in cold-acclimated plants. $n = 4$ for the *cor* genes; $n = 2$ for pHH25. Two of the determinations for the *cor* genes and one for pHH25 were from the plants used in the transcription rate assays. The values for the *cor* genes are minimum estimates since they do not include determinations where transcripts were not detected in control plants (*i.e.* cases in which the increase was 'infinite').

Figure 2.3 A representative example of data from the nuclear runon assays. Runons: Nuclei were isolated from cold treated and control plants, and transcription was allowed to continue in vitro in the presence of ^{32}P-labled UTP. Equivalent number of counts from both acclimated (A), and nonacclimated (N), were used to probe slot blots of isolated insert of the various *cor* genes and the gene for the small subunit of Rubisco (*rbcS*) as a control. Northerns: Total RNA was isolated from the same batch of plants from which the given nuclei preparations were made. RNA was run out on a formaldehyde agarose gel, blotted to Nytran, and hybridized to ^{32}P-labled insert DNA from the various *cor* genes and the *rbcS* gene.

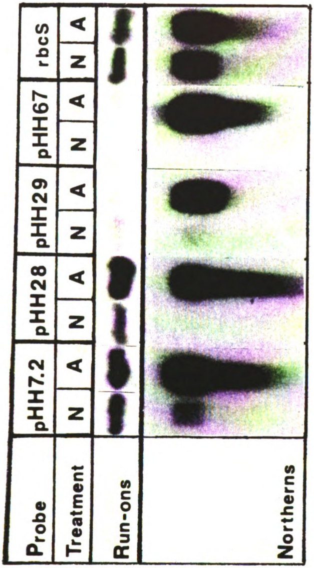

Probe
Treatment
Run-ons
Northerns
pHH7.2
pHH28
pHH29
pHH67
rbcS
N
A
N
A
N
A
N
A
N
A

Figure 2.3

Figure 2.4 Effect of drought treatment on accumulation of *cor* gene mRNA. Drought treatment was accomplished by withholding water from the plants. The relative water contents (RWC) of the plants were measured. Total RNA was extracted from the plants and 10 μg from each sample was fractionated on a formaldehyde gel and hybridized to the ^{32}P-labled isolated inserts from the indicated plasmids.

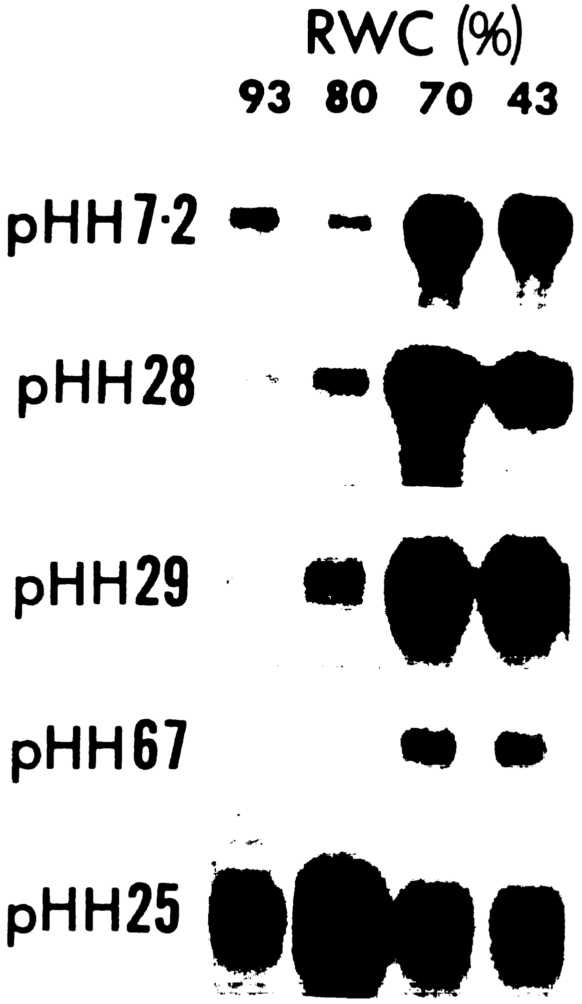


Figure 2.4

Because both drought stress and cold stress cause osmotic perturbations, the question arose as to if any osmotic stress could cause an increase in accumulation of *cor* gene message. To study this possibility, plants were watered with a 400 mM NaCl solution. Plants were visibly wilted after 24 hours. These plants were then harvested and their RNA extracted. Northern analysis was done to determine the amount of *cor* gene mRNA accumulated in response to the salt stress (Figure 2.5). The results demonstrated that the mRNA from the genes represented by pHH28, pHH7.2, and pHH29 all accumulate in response to salt stress. pHH67 mRNA however, accumulates very little in response to the salt treatment.

Because the *cor* genes are induced by drought as well as by cold, it was of interest to determine if the accumulation of the cold-regulated RNAs was simply the result of a general stress phenomenon. To check this possibility, plants were placed at 37°C (a temperature shown to be capable of inducing a heat shock response in *Arabidopsis*, (Wu *et al.*, 1987) for three to four hours, RNA was extracted, and accumulation of the four *cor* mRNAs was determined by northern analysis. The results from this experiment show that none of these genes are induced by heat shock (Figure 2.6); however, the conditions used for these heat shock treatments were sufficient to induce the gene for *hsp20*.

In addition to heat shock, plants were wounded by dicing with a razor, and floated on water for seven hours. Again, plants were harvested and the level of accumulation of *cor* gene mRNA was determined by northern analysis (Figure 2.7, lane 1). It was determined that the wounding treatment did not result in accumulation of *cor* gene mRNAs to levels higher than that of the unwounded, warm controls. Also, plants were wounded and floated on water at 3°C for seven

Figure 2.5 Effect of salt stress on *cor* gene mRNA accumulation. Plants were grown in 1 Liter clay pots in Bacto Potting Mix for 18 days. Plants were then watered with 200 mls of a 0.4 M NaCl solution (salt), or were placed at 5°C overnight (cold), or were left untreated (warm). After 24 hours, plants were harvested and total RNA was extracted for northern analysis. RNA was fractionated on formaldehyde agarose gels, hybridized to polyester backed nitrocellulose, and hybridized to the various *cor* genes.

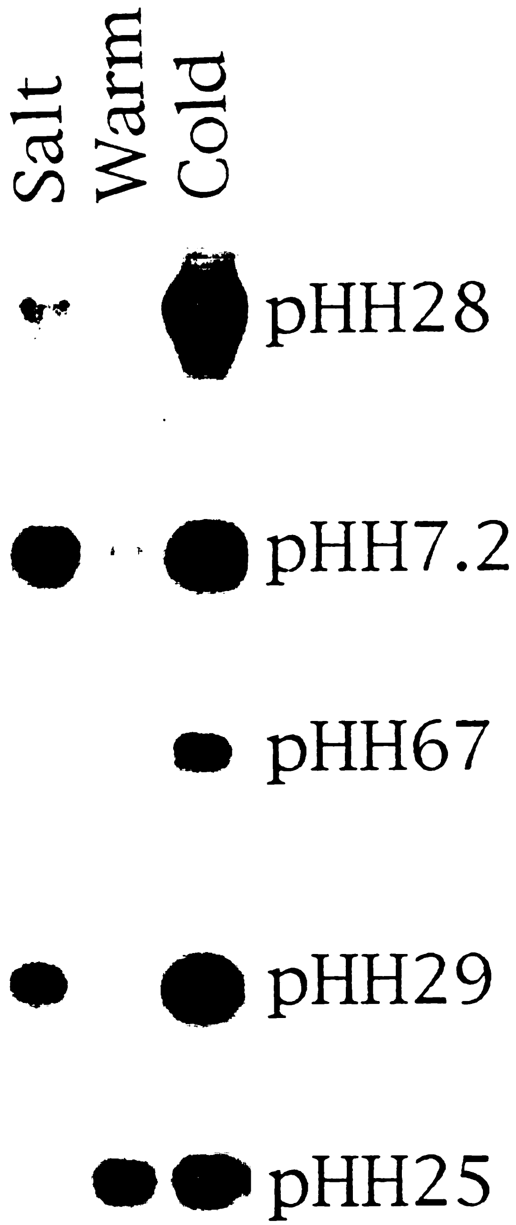


Figure 2.5

Figure 2.6 Effect of heat shock on *cor* gene message accumulation. Plants were placed at 37°C for 3 hours, and then harvested for total RNA extractions. RNA was fractionated on formaldehyde agarose gels, hybridized to polyester backed nitrocellulose, and hybridized to the various *cor* genes.

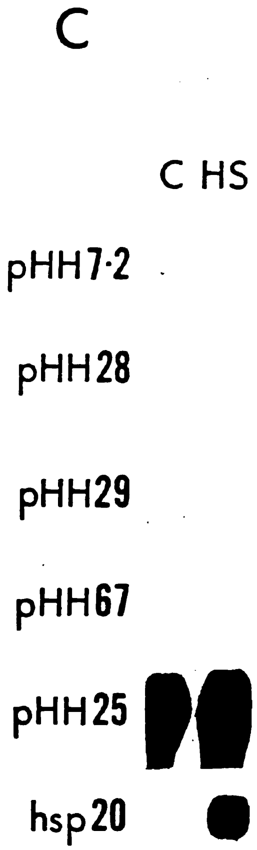


Figure 2.6

Figure 2.7 The effect of wounding on accumulation of *cor* gene mRNA. Plants were diced with a razor blade and floated on water for seven hours at 22^{o}C (warm/wounded) or at 3^{o}C (cold/wounded) or were left untreated (warm), or were placed at 3^{o}C overnight (cold). Plants were harvested and total RNA was extracted for northern analysis. RNA was fractionated on formaldehyde agarose gels, hybridized to polyester backed nitrocellulose, and hybridized to the various *cor* genes.

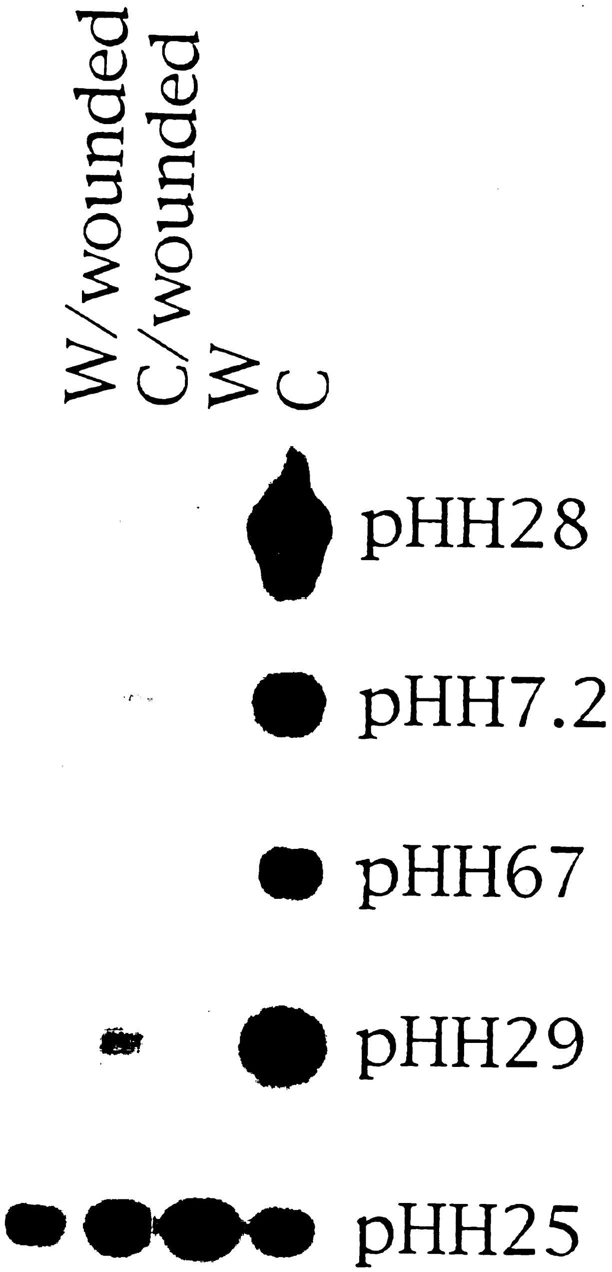


Figure 2.7

hours in order to determine that the wounding treatment did not prevent accumulation of *cor* gene message. The results from this treatment demonstrated that the wounded plants were still capable of inducing all of the *cor* genes except the one represented by pHH67 (Figure. 2.7, lane 2). The wounding and heat shock experiments support the idea that the *cor* genes are not induced by general plant stress, but are specifically induced by stresses related to cold.

Induction of the *cor* genes in roots, stems, and leaves and at various stages in the life cycle of *Arabidopsis thaliana*

The tissue specificity of *cor* gene mRNA accumulation was looked at by northern analysis. Four week old flowering plants were cold treated or left as untreated controls. Plants were harvested and plant parts were separated into roots, stems and flowers, and leaf rosettes, and RNA was extracted. The results of these northerns (Figure 2.8) indicate that the gene represented by pHH28 and pHH29 accumulate in all of the plant parts tested. The gene represented by pHH67 however, was only expressed in green tissues. Recent results have indicated that the gene represented by pHH67 (*cor15*) is a chloroplast targeted protein (Lin and Thomashow, 1992). Thus, it is not surprising that it would only be expressed in green tissue.

In addition to looking at tissue specificity, the ability of Arabidopsis to express these *cor* genes at various stages of their life cycle was examined. To do this, plants were cold treated at two weeks of age (which correlated to the 6 leaf stage), three weeks of age (when plants were just beginning to bolt), and at four weeks of age (when the plants were flowering and setting seed). The rosette portion of the plants were harvested, and RNA was extracted for northern analysis. The results of these experiments indicated that all of the *cor* genes tested accumulated in response to cold at all of the ages tested (Figure 2.9).

Figure 2.8 The tissue specificity of the accumulation of *cor* gene mRNA was examined. Flowering, pot grown *Arabidopsis thaliana* were left at 22°C (W for warm control) or were placed at were placed at 5°C for three days (C for cold treated). After treatment, Whole plants were harvested (total) or were separated into roots (R), stems (S), and leaves (L). RNA was extracted, and 10 ug of total RNA was subjected to northern analysis. Blots were probed with nick translated Blue Script plasmid containing cDNA clones of the *cor* genes tested.

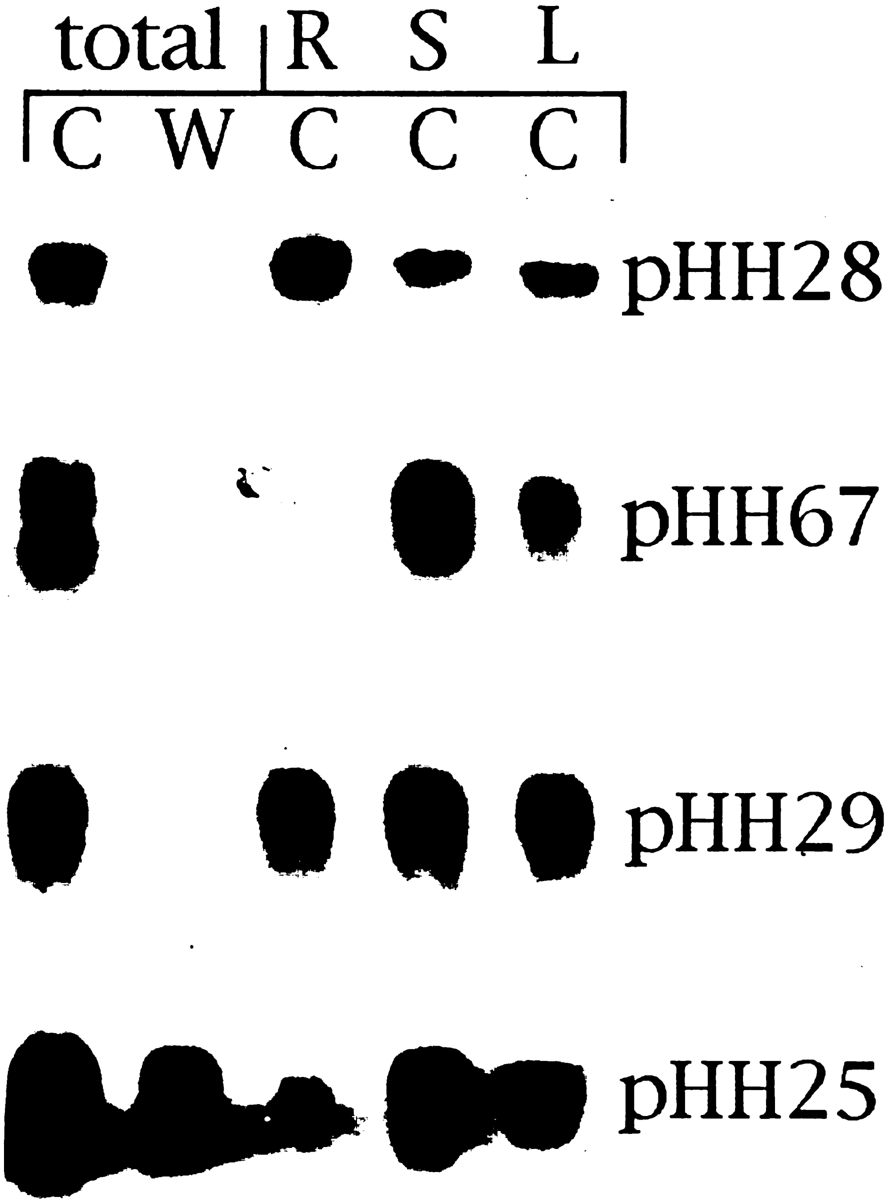


Figure 2.8

Figure 2.9 The ability of *Arabidopsis* to express the *cor* genes in response to cold throughout its life cycle was examined. Plants were harvested at 2 weeks, 3weeks, and 4weeks of age, before (warm), or after (cold) a cold treatment. RNA was extracted, and 10 ug of total RNA was subjected to northern analysis. Blots were probed with nick translated blue script plasmid containing cDNA clones of the *cor* genes tested.

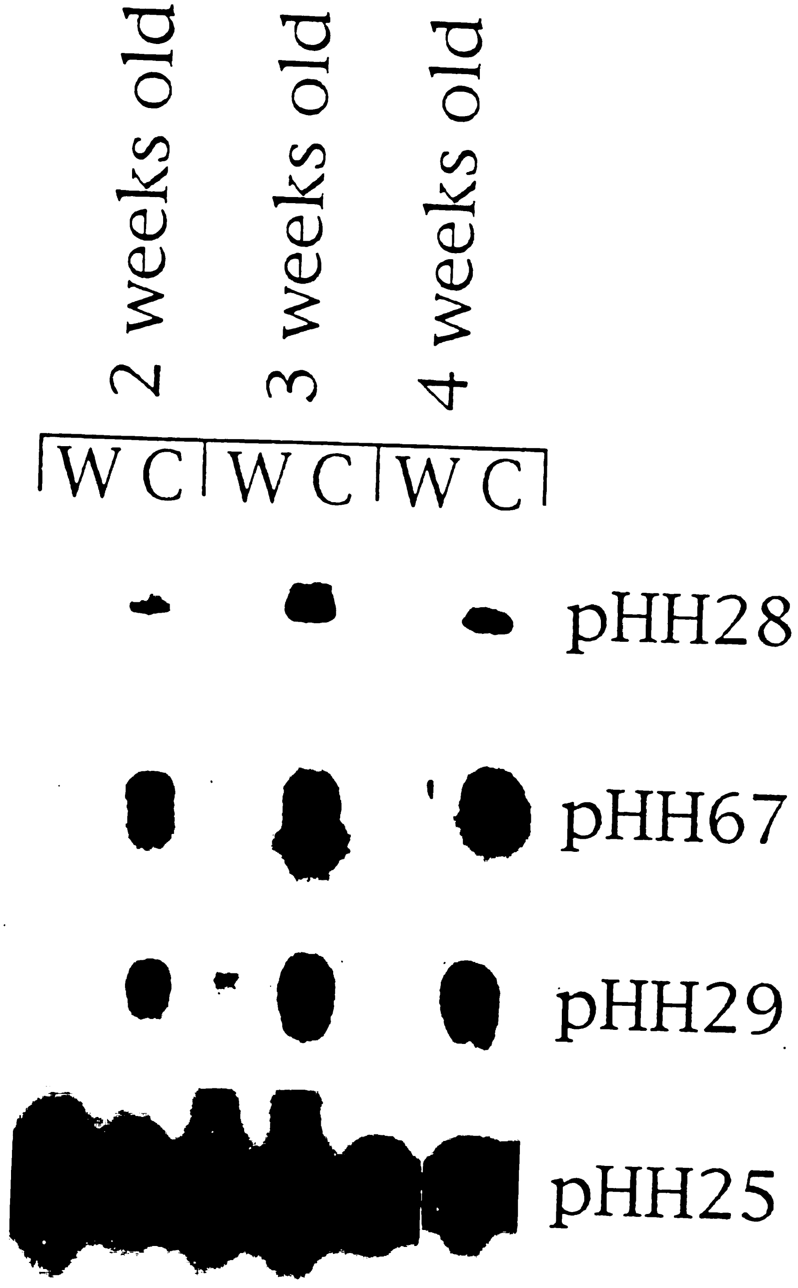

2 weeks old
3 weeks old
4 weeks old
W
C
W
C
W
C
pHH28
pHH67
pHH29
pHH25

Figure 2.9

DISCUSSION

Cold acclimation in higher plants is a many-faceted phenomenon. The most distinct manifestation of cold acclimation is increased freezing tolerance. To achieve this important low temperature survival ability, the plants must make numerous physiological changes that are likely to be mediated by the coordinated expression of several genes. It has been found that the rapid cold acclimation in *Arabidopsis thaliana* is concurrently accompanied by changes in steady state levels of a subset of translatable mRNAs (Gilmour *et al.*, 1988; Kurkela *et al.*, 1988). *In vivo* polypeptide labeling experiments have demonstrated that the protein products of many of these mRNAs also accumulate at the whole plant level. These observations, together with those in other plants, (Graham and Patterson, 1982; Cloutier, 1983, 84; Guy and Haskell 1989; Kurkela *et al.*, 1988; Gilmour *et al.*, 1988) clearly demonstrate selectively regulated gene expression during cold acclimation, and suggest a possible role for these genes in the process of cold acclimation. With the goal being to understand the molecular genetic basis of this process, isolation and molecular cloning of cold-regulated genes was a very important first step.

Currently, it has not been demonstrated that the *cor* genes play any role in the cold acclimation process. However, their cloning has provided additional evidence that the proteins they encode may be important in protecting the plant from freezing damage. Perhaps the strongest evidence for this comes from the fact that the *cor* genes have been evolutionarily conserved and their respective homologues all appear to be induced upon cold-stress (Guo *et al.*, 1992; Singh *et al.*, unpublished data). It has been shown that several of the *cor* genes have homologues in *Brassica napus* (Singh *et al.*, unpublished data), and one *cor* gene (*cor47*) has significant homology to a cold-regulated gene from wheat (Guo *et al.*, 1992). Also, all of the proteins encoded by the *cor* genes share two rather peculiar physical properties. They all remain soluble after boiling (Lin *et al.*, 1991), and all are extremely hydrophilic (Gilmour *et al.*, 1990; Horvath, see Chapter 3). Finally, another set of hydrophilic boiling soluble proteins from spinach were shown to protect isolated thylakoid membranes from freezing damage in *in vitro* assays (Volger and Heber, 1975). Thus, it seems unlikely that the *cor* genes would be conserved over great physiological distances (i.e. monocots to dicots) or that they would share similar structural motifs if they were not important. Also, since the *cor* genes and their homologues are cold-regulated, it implies that it is advantageous for plants to express proteins with similar physical characteristics during cold stress.

Along with expression during cold stress, the *cor* genes are also induced by osmotic stresses (such as drought and salt stress), and by exogenous application of abscisic acid (ABA) (Figures 2.4 and 2.5; Hajela *et al.*, 1990; Kurkula *et al.*, 1989). These data, though interesting, are not surprising given earlier findings that both drought stress and exogenous application of ABA have been shown to cause the accumulation of a similar subset of novel proteins (Reviewed by Guy, 1990). ABA and drought stress have been shown to increase the freezing tolerance of otherwise unacclimated plants (Chen *et al.*, 1983;Lang *et al.*, 1989). Finally, severe dehydration is one of the major causes of cellular damage during a freezing stress (Steponkus, 1984). Thus, there appears to be a common link between cold stress and drought stress.

ABA has been shown to increase in several plants (including *Arabidopsis*) in response to both drought and cold stress (Chen *et al.*, 1989; Palva *et al.*, personal communication). Also, ABA has been shown to increase freezing tolerance when exogenously applied to some plants (Chen *et al.*, 1989; Lang *et al.*, 1989). Therefore, it is tempting to speculate that ABA is the primary signal involved in regulating *cor* gene expression. There are several lines of evidence which suggest that drought, cold and ABA may all regulate *cor* gene expression via independent signal transduction pathways. It is known that *Arabidopsis* plants with a mutation that renders them insensitive to exogenous applications of ABA (designated *abi1* for ABA insensitive 1), were either cold treated, drought stressed, or sprayed with ABA, and the induction of the *cor* genes was monitored. The results showed that the *cor* genes were still capable of responding to drought and cold stress, but were not induced by exogenous application of ABA (Nordin *et al.*, 1991; Gilmour and Thomashow, 1991). Since initial analysis of the *abi1* mutation has shown that the exogenously applied ABA does accumulate in such plants (suggesting that these plants are not deficient in ABA uptake), these studies appear to indicate that the induction of the *cor* genes in response to both cold and drought is not dependent on the plant's ability to perceive increases in internal ABA levels (Finkelstein and Sommerville, 1990). Such results point to the possibility that the ABA response operates via a different (or partially different) signal transduction pathway than does the drought or cold responses. It is noted however, that since the nature of the *abi1* mutation is not known, it is possible that endogenous ABA may still be functioning as an intermediate, and that some part of the pathway involved with the transmission of signal from exogenously applied ABA may be interrupted in the *abi1* mutants. Since the exact nature of the signal transduction pathway(s) is not yet known, it would be interesting to study the *cis* acting regulatory sequences of these (and other) *cor* genes in order to isolate sequences that can impart specifically cold, ABA, or drought regulation to a reporter gene.

One of the observation that was made during the general characterization of the environmental parameters of the cold-induced accumulation of several of these cor genes was that they appeared to be induced at temperatures between $15^{o}C$ and $10^{o}C$. Interestingly, there are a number of other physiological processes that also demonstrate a temperature threshold within this range, including vernalization (Lang and Melchers, 1947) and stratification (Berrie, 1985). The membranes of several chilling sensitive plants have also been observed to undergo a phase shift from a flexible liquid crystalline state to that of a solid-gel structure at temperatures ranging from 12°C to 12°C (Lyons, 1973). This last observation is particularly interesting since it means that the plasma membrane may provide a physical mechanism for initiating a signal transduction pathway that could result in changes in gene expression. Thus, it is possible that changes in the state of the plasma membrane could be the primary sensor for low temperature sensing in plants.

The results of the nuclear runon experiments have suggested that the *cor* gene represented by pHH67 was primarily regulated at the transcriptional level (Figure 2.3). These data then suggest that the cold regulatory cis acting sequences in this gene should be found within its 5' nontranscribed sequences. In fact, there are recent data that support this conclusion. Constructs have been made that place the promoter for the gene represented by pHH67 in front of a GUS reporter gene. These constructs demonstrate the induction of the GUS reporter gene in response to cold, drought, and ABA thus confirming the implications of the nuclear runon assays (Baker, manuscript in preparation).

Nuclear runon results from the other three *cor* genes (represented by clones pHH28, pHH7.2, and pHH29) suggest that these genes are post-transcriptionally regulated (Figure 2.3). This would suggest that the *cis*-acting sequences responsible for the cold-regulation of these genes should be found within their transcribed regions. Sequence analysis of the cDNAs from these three *cor* genes suggests a number of interesting sequence motifs that may play a role in their regulation. The 3' untranslated regions (UTR) of all three of these presumably post-transcriptionally regulated *cor* genes are very "U" rich. Stretches of poly "U"s in the 3' UTR of several genes have been shown to be sites of nucleolytic cleavage in both *in vitro* and *in vivo* systems (Albrecht *et al.*, 1984; Ross, 1989). Also, the sequence AUUUA (of which there are several within the 3'UTR of the genes represented by pHH7.2 and pHH28) in the 3' UTR of several post-transcriptionally regulated genes from animal systems (*c-myc* and *GM-CFS*) has been shown to act as a binding site for proteins implicated in altering the stability of these (and other) mRNAs (Shaw and Kamen, 1986; Myer *et al.*, 1992).

However, it should be noted that nuclear runon assays have been known to give results that are difficult to interpret (Thompson and White, 1991). Consequently, more work will need to be done in order to be certain that the *cor* genes represented by pHH28, pHH29, and pHH7.2 are truly post-transcriptionally regulated. If these genes are truly post-transcriptionally regulated, then the sequences causing these genes to be cold-regulated should be in their transcribed region. Therefore, fusion constructs that place the transcribed portion of these genes under the control of a constitutive promoter, should be expressed in response to cold. Likewise, if these genes are really transcriptionally regulated, then the promoters of these genes should contain the necessary cis acting elements for cold-regulation. Thus, as was the case for the gene represented by pHH67, the promoters of these genes should be able to drive cold-regulated expression of a reporter gene.

Acknowledgments

I would like to credit and thank Dr. Ravindra Hajela for preparing and helping screen the cDNA library from which the *cor* genes were cloned. I would also like to thank him for preparation of figure 2.1.

Literature Cited

Albrecht G, Krowczynska A, Brawerman G (1984) Configuration of B-globin messenger RNA in rabbit reticulocytes. J. Mol. Biol. 178, 881-896

Ausubel FM, Brendt R, Kingston RE, Moore DD, Seidmon JG, Smith JA, Struhl K (1987) Current protocols in molecular biology. Greene Publishing Associates, Wiley Interscience, New York

Berrie, (1985) Germination and dormancy. *In* Advanced Plant Physiology M.B. Wilkins ed. Pitman Publishing Inc. Massechusetts

Chen HH, Gavinlertvatana P, Li PH (1979) Cold acclimation of stem cultured plants and leaf callus of solanum species. Bot. Gaz. 140, 142-147

Chen THH, Gusta LV (1983) Abscisic acid-induced freezing resistance in cultured plant cells. Plant Physiol. 73, 71-75

Chen HH, Li PH, Brenner ML (1983) Involvement of abscisic acid in potato cold acclimation. Plant Physiol. 71, 362-365

Cloutier Y (1983) Changes in the electrophoretic patterns of the soluble proteins of winter wheat and rye following cod acclimation and desiccation stress. Plant Physiol. 71, 400-403

Daie J, Campbell WF (1981) Response of tomato plants to stressful temperatures. Plant Physiol. 67, 26-69

Feinbaum RL, Ausubel FM (1988) Transcriptional regulation of the *Arabidopsis thaliana* chalcone synthase gene. Mol. Cell. Bio.. 8, 1985-1992

Freier S, Kierzek R, Jeager J, Sugimoto N, Caruthers M, Neilsin T, Turner D (1986) Improved free energy parameters for predictions of RNA duplex stability. PNAS 83, 9373-9377

Gilmour SJ, Hejela RK, Thomashow MF (1988) Cold acclimation in *Arabidopsis thaliana*. Plant Physiol. 87, 745-750

Graham D, Patterson BD (1982) Responses of plants to low, non-freezing temperatures: proteins, metabolisms, and acclimation. Annu. Rev. Plant Physiol. 33, 347-372

Gulber U, Hoffman BJ (1983) A simple and very efficient method for generating cDNA libraries. Gene 25, 263-269

Guo W, Ward R, Thomashow M (1992) Characterization of a cold-regulated gene from wheat related to *Arabidopsis cor47*. Plant Physiol. 100, 915-922

Guy CL, Haskell D (1989) preliminary characterization of high molecular mass proteins associated with cold acclimation in spinach. Plant Physiol. Biochem. 27, 389-394

Guy CL, Haskell D, Yelenosky G (1988) Changes in freezing tolerance and peptide content of spinach and citrus at 5^{o}C. Cryobiology 25, 264-271

Guy C (1990) Cold acclimation and freezing stress tolerance: role of protein metabolism. Ann. Rev. of Plant Physiol. 41, 187-223

Kasperska-Palacz A , Dlugokecka E, Breitenwald J, Wcislinska B (1977) Physiological mechanisms of frost tolerance: possible role of protein in plant adaptation to cold. Biol. Plant. 19, 10-17

Keith CN, McKensie BD (1986) The effect of abscisic acid on the freezing tolerance of callus cultures of *Lotus cornicalatus* L. Plant Physiol. 80, 766-770

Koeller D, Casey J, Hentze M, Gerhardt E, Lee-Nien C, Klausner R, Harford J (1989) A cytosolic protein binds to structural elements within the iron regulatory region of the transferrin receptor mRNA. PNAS 86, 3574-3578

Kurkela S, Franck M, Heino P, Lang V, Palva T (1988) Cold-induced gene expression in *Arabidopsis thaliana* L. Plant Cell Rep. 7, 495-498

Lang A. and Melchers G. (1947) Vernalization und devernalization bei einer zweijahrigen pflanze. Z. Naturf. 2, 444-449

Lang V, Heino P, Palva ET (1989) Low temperature acclimation and treatment with exogenous abscisic acid induce common polypeptides in *Arabidopsis thaliana* (L.) Heynh. Theoretical and Applied Genetics 77, 729-734

Levitt J (1980) Responses of plants to environmental stress. Chilling, freezing, and high temperature stresses. Ed 2, Academic Press, New York

Lyons J.M. (1973) Chilling injury in plants. Ann. Rev. Plant Physiol. 24, 445-466

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Meyerowitz EM (1989) *Arabidopsis*, a useful weed. Cell 56, 263-269

Mullner E, Kuhn L (1988) A stem loop in the 3' untranslated region mediates iron dependent regulation of the transferrin receptor mRNA stability in the cytoplasm. Cell 53, 815-825

Olien CR (1977) Ice adhesions in relation to freezing stress. Plant Physiol. 60, 499-503

Orr W, Keller WA, Singh J (1986) Induction of freezing tolerance in an embryonic cell suspension culture of *Brassica napus* by abscisic acid at room temperature. J. Plant Physiol. 126, 23-32

Reaney MJT, Gusta LV (1987) Factors influencing the induction of freezing tolerance by abscisic acid in cell suspension cultures of *Bromus inermis* Leyss and *Medicago sativa* L. Plant Physiol. 83, 423-427

Rikin A, Waldman M, Richmond AE, Dovrat A (1975) Hormonal regulation of morphogenisis and cold-resistance. J. Exper. Bot. 26, 175-183

Ross J (1989) The turnover of messenger RNA. Scientific American Apr. pp48-55

Sakai A, Larcher W (1987) Frost survival of plants: response and adaptation to freezing stress. Spring-Verlag, New York

x Shaw G, Kamen R (1986) A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell 46, 659-667

Thomashow M (1990) Molecular genetics of cold acclimation in higher plants. In Scandalios JG, and Wright TRF, eds. Advances in genetics, Vol. 28, Genetic Responses to Environmental Stress. Academic Press, New York

Thomashow M, Gilmour S, Hajela R, Horvath D, Lin C, Guo W (1990) Studies on cold acclimation in *Arabidopsis thaliana*. IN, AB Bennett, SD O'Neill, eds, Horticultural Biotechnology. Wiley-Liss, New York, pp305-314

Thompson EF, White MJ (1991) Physiological and molecular studies of light-regulated nuclear genes in higher plants. Ann. Rev. Plant Physiol. 42, 423-466

Trunova TI (1982) Mechanisms of winter wheat hardening at low temperature . *In* PH Li, A Sakai, eds, Plant cold hardiness and freezing stress, Vol 2. Academic Press, New York, pp 41-54

Volger HG, Heber U (1975) Cryoprotective leaf proteins. Biochem. Biophys. Acta. 412, 335-349

Wilson CC (1948) The effect of some environmental factors on the movements of guard cells. Plant Physiol. 23, 5-18

Wright STC, Hiron RWP (1972) The accumulation of abscisic acid in plants during wilting and under other stress conditions. *In* DJ Can, ed., Plant Growth Substances. New York Springer-Verlag.

Chapter 3

Regulation of the *Arabidopsis cor78* gene

SUMMARY

In order to gain a better understanding of the regulation of the apparently post-transcriptionally regulated *cor* genes from *Arabidopsis*, the genomic clone of the *cor78* gene was obtained and a portion of it sequenced. The 5' portion of this clone was used as a probe to isolate a near full length cDNA clone corresponding to this gene. Sequence analysis of both clones indicated that this gene has three introns and should encode a highly hydrophilic, 79 kD polypeptide. In vitro transcription and translation of the near full length cDNA, however, demonstrated that the protein encoded by *cor78* runs as 160 kD on an SDS-PAGE gel. A series of constructs containing portions of the genomic clone were prepared to determine which regions of the *cor78* gene were responsible for its cold-regulated expression. Analysis of the expression of these constructs in transgenic *Arabidopsis* indicated that sequences responsible for the cold-regulation of the *cor78* gene are contained within its promoter. Implications of this result with regards to previous nuclear runon data are discussed. Histological staining for *cor78* promoter driven GUS expression demonstrated that the *cor78* promoter is active throughout most of the plant during cold treatment. Experiments that followed the kinetics of *cor78* induction and repression during and after a three hour cold treatment demonstrated a lag period of approximately two hours prior to the

induction and 45 min prior to the inactivation of the *cor78* gene. The implications for the mechanisms involved in signal transduction are discussed.

INTRODUCTION

Many plants indigenous to temperate regions of the earth are exposed to extremes in low temperature. Thus, it is not surprising that some plants have evolved means by which they can sense temperature drops and alter their biochemistry in order to prevent or minimize damage caused by freezing. In fact it is known that many plants, if first subjected to low but non-freezing temperatures, are able to become more tolerant to freezing stress (Levitt, 1980), a process called cold acclimation.

Much is known about the changes in plant biochemistry that occur during the cold acclimation process. Many plants show a buildup of small soluble molecules such as proline and sugars (Sakai and Larcher, 1987). Often changes in the composition of membrane lipids also accompany the plant's response to the cold (Steponkus *et al.*, 1988). Finally, there are data to suggest that protein synthesis is necessary for the cold acclimation process to occur (Kasperkas-Palacz *et al.*, 1977). In fact, there are specific changes in the protein profile of plants that have undergone cold acclimation (reviewed in Thomashow, 1990).

To gain a better understanding of the molecular basis of cold acclimation, four genes from *Arabidopsis thaliana* that encode proteins which accumulate specifically in plants undergoing cold acclimation have been recently cloned. These four cold-regulated (*cor*) genes, designated *cor6.6*, *cor15*, *cor47*, and *cor78* (previously referred to as genes represented by clones pHH29, pHH67, pHH7.2, and pHH28 respectively (Hajela *et al.*, 1990), all encode boiling soluble proteins that were observed to accumulate during conditions that induce cold acclimation (Lin *et al.*, 1990). Other environmental stimuli that affect their regulation have been studied, and it has been learned that many of these *cor* genes can be induced by ABA and by osmotic stress (Hajela *et al.*, 1990). Also, work using ABA deficient and insensitive mutants has demonstrated the possibility for separate but converging signal transduction pathways in the regulation of *cor* genes by these environmental stimuli (Gilmour *et al.*, 1991; Nordin *et al.*, 1991). However, although there is growing evidence on the signal transduction pathways that operate to alter the expression of these genes, little is known about the location of sequences within these genes that are necessary for its regulation. Nuclear runon assays have indicated that at least one *cor* gene, *cor15*, is primarily transcriptionally regulated, suggesting that the sequence(s) important for its regulation is located within this gene's promoter (Hajela *et al.*, 1990). This hypothesis was recently confirmed by promoter fusion studies (Baker *et al.*, manuscript in preparation). Nuclear runon assays also indicated that *cor6.6, cor47,* and *cor78* are primarily post-transcriptionally regulated. As such, it would be predicted that the important *cis*-acting elements will be found in the transcribed portion of these genes (Hajela *et al.*, 1990).

In order to learn more about the nature of the *cis*-acting sequences within *cor* genes and to gain insight into the signal transduction pathways that may function in their regulation, a genomic and a near full length cDNA clone of the *Arabidopsis cor78* gene have been cloned and characterized. Further, the kinetics of *cor78* transcript accumulation under inducing and repressing conditions were a studied. In order to localize the important *cis*-acting elements, constructs using these clones and the GUS reporter gene plasmids developed by Jefferson *et al.* (1987) have been prepared and their expression followed in transgenic *Arabidopsis*. The GUS reporter gene was used to determine tissue and cell type specificity for one of the cold-regulated constructs in the transgenic plants.

MATERIALS AND METHODS

Plant Material and Treatments

Arabidopsis thaliana L. (Heyn) ecotypes RLD and Landsberg erecta were generally grown in pots for 2 to 3 weeks in controlled environment chambers at 21°C under constant light (approximately 100 umole m^{-2} s^{-1}). For most cold-regulation experiments, plants were transferred to a controlled environment chamber at 5°C (constant light) for various lengths of time. For ABA, drought and some cold treatments, plants were grown in petri plates on germination medium (GM) supplemented with B-5 vitamins (Valvekes *et al.*, 1988) at room temperature (about 25°C) on a 16 hour day 8 hour night schedule until they reached the 4-6 leaf stage (about two weeks). ABA treatment consisted of plants being sprayed to runoff with 100 uM ABA (mixed isomers, Sigma) in 0.02% (v/v) Tween-20; control plants were sprayed with a solution of 0.02% (v/v) Tween-20. Treated plants were covered to slow evaporation and placed on the lab bench for 4 hours. Drought stress treatments consisted of removing the lid of the petri dish and allowing the plants to dry overnight in the growth chamber, at which point they had become visibly wilted. In the time course cold-regulation studies, *A. thaliana* ecotypes Landsberg and RLD were grown on petri plates as described above. Plates were then transferred to a cold room at 2°C with constant light (approximately 50 umole m^{-2} s^{-1}) for various lengths of time.

Isolation and Analysis of Nucleic Acids

Plant material was frozen in liquid nitrogen, pulverized using a mortar and pestle, and stored at -80°C prior to extraction of nucleic acids. Total and poly(A^{+}) RNA and genomic DNA was isolated as previously described (Hajela *et al.*, 1990). Plasmid

DNA was prepared from *E. coli* using standard protocols (Maniatis *et al.*, 1982). Northern transfers were prepared and hybridized with [^{32}P]-labeled probes as described previously (Maniatis *et al.*, 1982).

Isolation and DNA Sequencing of Genomic and cDNA Clones for *cor78*

The insert from cDNA clone pHH28 (Hajela *et al.*, 1990) was gel purified (Sambrook *et al.*, 1989), radiolabeled with ^{32}P and used to screen plaque lifts (Sambrook *et al.*, 1989) of a lambda EMBL3 library of *A. thaliana* genomic DNA (a SauIIIa partial digest of ecotype Columbia; the library was kindly provided by Dr. Harry Klee, Monsanto) for genomic clones carrying *cor78*. One such clone, lambda 28-4, was isolated and an 8.4 kb HindIII fragment (containing 4.4 kb of lambda sequences and 4 kb of *Arabidopsis* sequences including the entire transcribed portion of *cor78* as well as 808 bps of 5'upstream sequences) was subcloned into bluescript. This clone was then cut with SalI which liberated two 2kb fragments corresponding to the 5' and 3' halves of the *cor78* gene. Both fragments were subsequently cloned separately into bluescript and designated pDH28-5' and pDH28-3' respectively. A gel isolated insert from pDH28-5' was then used to generate a probe for screening a cDNA library (in lambda ZAP) of *A. thaliana* (Lin and Thomashow, 1992) for clones that contained near full-length inserts. One chosen for further study contained 2588 bps from +19 to +2607. This recombinant cDNA was 'excised' out of the phage in plasmid bluescript SK$^-$ using the biological rescue recommended by Stratagene, thus creating plasmid pBM1. DNA sequencing was done by the dideoxy method of Sanger *et al.*, (1977) using double-stranded DNA templates as described previously (Lin and Thomashow 1992). Hydropathy plots were conducted using a window of nine amino acid residues according to Kyte and Doolittle (1982). TFASTA and WORDSEARCH programs were used to search protein and nucleic acid data bases (EMBL, GenBank, PIR, and NBRF) for previously sequenced proteins and genes.

In Vitro Transcription/Translation Reactions

pBM1 was linearized by digestion with BamHI and the insert was transcribed *in vitro* with T7 RNA polymerase (Promega) using the T7 promoter carried on the bluescript vector. The resulting transcripts were extracted with PCI (phenol:chloroform:isoamyl alcohol (25:24:1)) and precipitated with ethanol. Transcription products from these reactions and total RNA isolated from cold-treated and control *Arabidopsis thaliana* ecotype RLD were translated *in vitro* using the rabbit reticulocyte lysate system (Promega) containing [^{35}S]methionine. Boiling-soluble polypeptides were prepared as described (Lin *et al.*, 1990), and were fractionated by SDS-PAGE (Laemmli, 1970) on 15% (w/v) polyacrylamide gels and visualized by autoradiography.

Primer Extension Analysis

Primer extension experiments were done according to Sambrook *et al.*, (1989) with modifications. In a total reaction volume of 30 ul, 100 ug of total RNA from cold-treated (15 hour) *A. thaliana* ecotype RLD was mixed with 8 ul of 5X M-MLV reverse transcriptase buffer (BRL), and 1 ul of a 0.02 nM stock of a 21 bp primer corresponding to bp +39 to +59 that had been end-labelled with ^{32}P (1-2 x 10^5 cpm) (Sambrook *et al.*, 1989). The mixture was heated to 70^{o}C for 10 min and placed at room temperature for 2 h. 10 ul of a 4X extension cocktail, consisting of 4 mM DTT, 2.4 mM each dNTP, and 0.2 units InhibitAce (5 Prime -> 3 Prime, Inc.), was added along with 30 units of M-MLV reverse transcriptase and the mixture was incubated for 10-20 min at 42^{o}C. The reaction was stopped by heating to 70^{o}C for 5 min followed by incubation with 10 ul RNase A at 42^{o}C for 30 min. The reaction was extracted twice with PCI and once with $CHCl_3$ and then precipitated with EtOH. The pellet was resuspended in 10 ul of loading buffer (50% formamide, 0.01% bromophenol blue, 0.01% xylene cyanol) and run on an 8% DNA sequencing gel. The gel was dried and autoradiographed for 24-48 hours with an intensifying screen at -80^{o}C.

cor78 Constructs and Plant Transformation

The construct *78Pgus* (pDH78P) contains the promoter region of *cor78* from bp -808 to bp +5 inserted into the polylinker of pBI101.1 (Jefferson *et al.*, 1987) in such a way as to drive the expression of the GUS reporter gene (see Figure 3.5A). It was made by PCR of the plasmid pDH28-5' using the T7 bluescript sequencing primer as the 5' primer. The synthesized 3' primer was designed to hybridize to bps +5 to -12, (a BamHI site was included at the 3' end of this primer in order to facilitate cloning into pBI101.1). This fragment was blunt ended by a Klenow reaction (Maniatis *et al.*, 1982) and sub-cloned into the SmaI site of bluescript. Finally, this fragment was cloned into pBI101.1 as a BamHI-HindIII fragment, and the junction was confirmed by sequencing using the GUS primer (Clontech Laboratories Inc.).

Construct *78PIgus* (pDH78PI) contains a portion of the *cor78* genomic clone from bp -808 to bp +250 ligated 5' to the GUS gene from pBI101.2 (Jefferson *et al.*, 1987) (see Figure 3.5A). This construct was designed to place the 5'portion of the *cor78* gene (as expressed from the *cor78* promoter) in the same reading frame as the GUS gene from pBI101.2. The 1058bp fragment of DNA from *cor78* (-808 to +250) was obtained by an exo-mung bean deletion of the 3' portion of the genomic clone from pDH28-5'. The deleted pDH28-5' was cut with SalI and the resulting fragment of *cor78* was ligated into SalI/SmaI cut bluescript (creating plasmid pDH 15K). pDH

15K was cut with BamHI and SalI and the gel purified insert was ligated into the SalI/BamHI cut polylinker of pBI101.2 to create pDH78PI.

Constructs were also made that contained portions of the *cor78-3'* transcribed region (oriented 5' to 3') ligated between the GUS gene from pBI131 and a nopaline synthase terminator sequence that contained all of the necessary signals for poly A tail addition (see Figure 3.6A). The plasmid pHH28 was subjected to exo-mung bean deletion at its 3' end, and the resulting fragments were religated in the presence of SacI linkers. This resulted in a pool of bluescript-derived plasmids that contained various deletions of the *cor78* gene with an added SacI site at their 3' end. The extent of the deletions from several of these plasmids was determined by sequencing. Two such plasmids had deletions ending at bp +2981 and +2983, thus removing the predicted poly A addition signals. One of these clones was cut with SacI thus releasing a 1000 bp fragment which was ligated into SacI cut pBI131 to create plasmid pDH410C. The orientation of the resulting clones was determined by restriction mapping. The other deleted plasmid was cut with AluI which cut the *cor78* sequence at bp +2877 leaving a blunt ended fragment containing the 3'untranslated region (UTR) of *cor78* and a portion of the bluescript plasmid. The fragment containing the 3'UTR of *cor78* was gel purified and subcloned into another bluescript plasmid that had been cut with SmaI creating plasmid pDH 3'. pDH 3' was then cut with SacI and the resulting 250bp fragment was also cloned into SacI-cut pBI131 creating the plasmid pDH81C. Again, the orientation of the resulting clones was confirmed by restriction mapping.

The construct containing the entire transcribed region of the *cor78* gene driven by the 35S CaMV promoter *35Scor78* (pDH78T) (see Figure 3.6B) was prepared in two steps. First, PCR was used to prepare a fragment containing all of the transcribed sequences of *cor78* from pDH28-5'. The 5' primer for the PCR reaction was designed to hybridize to bps -11 to +6 (and included an XbaI site at the 5' end to facilitate cloning into pBI121 (Jefferson *et al.*, 1987). The 3' primer was the T3 primer for bluescript. This PCR fragment was blunt ended by treatment with the Klenow fragment of *E. coli* DNA polymerase, and sub-cloned into SmaI cut bluescript. The PCR fragment was subsequently cloned into pBI121 as an XbaI/EcoRI fragment in such a way as to replace the GUS gene and the nopaline synthase terminator sequences. This construct was confirmed by sequencing across the junction between the CaMV promoter and the *cor78* transcriptional start site with the primer used in the primer extension assays described above. The 2 kb SalI fragment that contained the rest of the 3' portion of the gene along with approximately 400 bp of 3' untranslated sequences was cloned into the SalI site of the modified pBI121 plasmid. The orientation of the cloning was confirmed by restriction mapping of the new plasmid.

All constructs were transformed into *A. thaliana* RLD using the *Agrobacterium* mediated root transformation protocol (Valvekes *et al.*, 1988), with the exception that kinetin was omitted from the callus inducing medium (CIM) and -napthaleneacetic acid (NAA) was omitted from the shoot inducing medium (SIM).

RNase Protection Assays

The RNA probe (for the RNase protection assays) was prepared from band isolated pDH 15K which had been cut with MluI and SalI to linearize the plasmid and remove sequences 5' to bp -113. *In vitro* transcription of the probe was accomplished using the Maxi-script kit (Ambion Inc.) following the manufacturer's protocols. RNase protection assays were done with the Rapa II kit (Ambion Inc.) following the manufacturer's protocols.

Histochemical Staining for GUS Activity.

Plants transformed with the plasmids pDH78PI and pBI121 were grown in pots and cold-treated as described above. Whole plants or plant parts were then placed in GUS staining solution (100mM $NaPO_4$, 3mM $K_3[Fe(CN)_6]$, 10mM EDTA, 0.1% Triton X100, and 2mM 5-bromo-4-chloro-3-indoyl-ß-D-glucopyranoside (X-Gluc)). Samples were vacuum infiltrated in the GUS staining solution for at least 20 minutes and then incubated at 37^oC overnight.

For thin sections, plants were embedded in plastic (De Block and Debrouwer, 1992), and cut into 10 or 20 micron sections using a glass knife. GUS staining was done either before embedding (as described above), or after sectioning by placing the section between a glass slide and a cover slip and allowing a drop of GUS staining solution to seep between them and hydrate the section. The slides were then placed in a humidity chamber and incubated at 37^oC overnight.

RESULTS

Cloning and characterization of *cor78*

Previous work resulted in the cloning and characterization of pHH28 a partial cDNA for the *cor78* (previously *cor160*) gene (Hajela *et al.*, 1990). In order to learn more about the structure and regulation of the *cor78* gene, a cDNA clone (isolated by Brett McLarney, which appeared to contain all of the translated sequences), and a genomic clone of the *cor78* gene were isolated. Both clones were sequenced (Again with the help of Brett McLarney) (Figure 3.1). Analysis of the data indicated that this gene contained three short introns, (all less than 100 bps each) and a 68 bp 5'

Figure 3.1 Nucleotide sequence and predicted protein sequence of the genomic clone for *cor78* . Promoter sequences with probable functions are labeled and underlined. Transcribed but untranslated sequences are denoted in lower case letters. Repeated amino acid sequences within the predicted protein are underlined for repeat 1, double underlined for repeat 2, and bold typed and underlined for repeat 3. The probable poly A tail addition signal is underlined, and the beginning of the poly A tail as determined by sequences from two separate cDNA clones is labeled.

```
                              GATCTCAAAGTTTGAAAGAAAATTTATTTCTTCGACT  -772
CAAAACAAACTTACGAAATTTAGGTAGAACTTATATACATTATATTGTAATTTTTTGTAACAAAATG  -705
TTTTTATTATTATTATAGAATTTTACTGGTTAAATTAAAAATGAATAGAAAAGGTGAATTAAGAGGA  -638
GAGAGGAGGTAAACATTTTCTTCTATTTTTTCATATTTTCAGGATAAATTATTGTAAAAGTTTACAA  -571
GATTTCCATTTGACTAGTGTAAATGAGGAATATTCTCTAGTAAGATCATTATTTCATCTACTTCTTT  -504
TATCTTCTACCAGTAGAGGAATAAACAATATTTAGCTCCTTTGTAAATACAAATTAATTTTCGTTCT  -437
TGACATCATTCAATTTTAATTTTCGTATAAAATAAAAGATCATACCTATTAGAACGATTAAGGAGAA  -370
ATACAATTCGAATGAGAAGGATGTGCCGTTTGTTATAATAAACAGCCACACGACGTAAACGTAAAAT  -303
GACCACATGATGGGCCAATAGACATGGACCGACTACTAATAATAGTAAGTTACATTTTAGGATGGAA  -236
            Repeat
TAAATATCATACCGACATCAGTTTGAAAAGAAAAAGGGAAAAAAAGAAAAAATAAATAAAAGATATA  -169
     Repeat
CTACCGACATGAGTTCCAAAAAGCAAAAAAAAAGATCAAGCCGACACAGACACGCGTAGAGAGCAAA  -102
                                     ABRE(?)
ATGACTTTGACGTCACACCACGAAAACAGACGCTTCATACGTGTCCCTTTATCTCTCTCAGTCTCTC  -35
 TATA                              +1|->
TATAAACTTAGTGAGACCCTCCTCTGTTTTACTC acaaatatgcaaactagaaaacaatcatcagga  +33

ataaagggtttgattacttctattggatttggaa ATG GAT CAA ACA GAG GAA CCA CCA  +92
                                    M   D   Q   T   E   E   P   P
CTC AAC ACA CAC CAG CAG CAC CCA G gtagattctaatttcagaaacttatattttttt  +150
 L   N   T   H   Q   Q   H   P
taagtgacaatcctctgaatttacttaaacttattgtgatttatggatacag AA GAA GTT GAA  +213
                                                     E   E   V   E
CAT CAT GAG AAT.GGT GCG ACT AAG ATG TTT AGG AAA GTA AAG GCT AGA GCT  +264
 H   H   E   N   G   A   T   K   M   F   R   K   V   K   A   R   A
AAG AAG TTC AAG AAC AGT CTC ACT AAA CAT GGA CAA AGC AAT GAG CAT GAG  +315
 K   K   F   K   N   S   L   T   K   H   G   Q   S   N   E   H   E
CAA GAT CAT GAT TTG GTT GAA GAA GAT GAT GAT GAT GAC GAG CTA GAA CCT  +366
 Q   D   H   D   L   V   E   E   D   D   D   D   D   E   L   E   P
GAA GTG ATC GAT GCA CCA G  gttactttcttttgtagtttcattcaacttatgatctaaa  +425
 E   V   I   D   A   P
aactattggttattcaattttgcgtgacattaacggtttggtatctgtatatgcag GC GTA ACA  +489
                                                         G   V   T
GGT AAA CCT AGA GAA ACT AAT GTT CCA GCA TCG GAG GAA ATT ATT CCA CCA  +540
 G   K   P   R   E   T   N   V   P   A   S   E   E   I   I   P   P
GGG ACA AAG GTG TTT CCT GTC GTG TCT TCC GAT TAC ACC AAA CCC ACT GAA  +591
 G   T   K   V   F   P   V   V   S   S   D   Y   T   K   P   T   E
TCT GTA CCA GTA CAA GAG GCC TCT TAC GGA CAC GAT GCA CCG GCT CAT TCT  +642
 S   V   P   V   Q   E   A   S   Y   G   H   D   A   P   A   H   S
GTA AGG ACG ACG TTT ACA TCG GAC AAG GAA GAG AAA AGA GAT GTA CCG ATT  +693
 V   R   T   T   F   T   S   D   K   E   E   K   R   D   V   P   I
CAT CAT CCT CTG TCC GAA TTG TCA GAC AGA GAA GAG AGT AGA GAG ACT CAT  +744
 H   H   P   L   S   E   L   S   D   R   E   E   S   R   E   T   H
```

Figure 3.1

```
CAT GAG TCA TTG AAC ACT CCG GTC TCT CTG CTT TCT GGA ACA GAG GAT GTA   +795
 H   E   S   L   N   T   P   V   S   L   L   S   G   T   E   D   V
ACG AGT ACG TTT GCT CCA AGT GGT GAT GAT GAA TAT CTT GAT GGT CAA CGG   +846
 T   S   T   F   A   P   S   G   D   D   E   Y   L   D   G   Q   R
AAG GTC AAC GTC GAG ACC CCG ATA ACG TTG GAG GAA GAG TCG GCT GTT TCA   +897
 K   V   N   V   E   T   P   I   T   L   E   E   E   S   A   V   S
GAC TAT CTT AGT GGT GTA TCT AAT TAT CAG TCC AAA GTT ACT GAT CCC ACC   +948
 D   Y   L   S   G   V   S   N   Y   Q   S   K   V   T   D   P   T
AAA GAA G gtaagaactttgacctttaagattgtgtttttctttagtgattatgaatatgtaat   +1012
 K   E
aactctgttacgttgtgtttggtttag  AA ACT GGA GGA GTA CCG GAG ATT GCT GAG   +1068
                              E   T   G   G   V   P   E   I   A   E
TCT TTT GGT AAT ATG GAA GTG ACT GAT GAG TCT CCT GAT CAG AAG CCA GGA   +1119
 S   F   G   N   M   E   V   T   D   E   S   P   D   Q   K   P   G
CAA TTT GAA AGA GAC TTG TCG ACG AGA AGC AAA GAA TTC AAA GAG TTT GAT   +1170
 Q   F   E   R   D   L   S   T   R   S   K   E   F   K   E   F   D
CAG GAC TTT GAC TCT GTT CTC GGT AAG GAT TCG CCG GCT GAA ATT TCC AGG   +1221
 Q   D   F   D   S   V   L   G   K   D   S   P   A   K   F   P   G
GAA TCA GGA GTT GTT TTC CCG GTG GGC TTT GGT GAC GAG TCA GGA GCT GAG   +1272
 E   S   G   V   V   F   P   V   G   F   G   D   E   S   G   A   E
CTG GAA AAA GAT TTT CCG ACG AGA AGT CAT GAT TTT GAT ATG AAG ACT GAA   +1323
 L   E   K   D   F   P   T   R   S   H   D   F   D   M   K   T   E
ACT GGA ATG GAC ACG AAT TCT CCA TCA AGA AGC CAT GAA TTT GAT CTG AAG   +1374
 T   G   M   D   T   N   S   P   S   R   S   H   E   F   D   L   K
ACT GAA TCT GGA AAC GAC AAG AAT TCT CCG ATG GGC TTT GGT AGT GAA TCA   +1425
 T   E   S   G   N   D   K   N   S   P   M   G   F   G   S   E   S
GGA GCT GAG CTG GAA AAA GAA TTT GAT CAG AAG AAC GAT TCT GGA AGA AAC   +1476
 G   A   E   L   E   K   E   F   D   Q   K   N   D   S   G   R   N
GAG TAT TCG CCG GAA TCT GAC GGC GGT TTA GGA GCT CCG TTG GGA GGA AAT   +1527
 E   Y   S   P   E   S   D   G   G   L   G   A   P   L   G   G   N
TTT CCG GTG AGA AGT CAT GAG TTG GAT CTG AAG AAC GAA TCT GAT ATC GAC   +1578
 F   P   V   R   S   H   E   L   D   L   K   N   E   S   D   I   D
AAG GAT GTG CCG ACG GGA TTT GAC GGA GAA CCA GAT TTT CTG GCG AAG GGA   +1629
 K   D   V   P   T   G   F   D   G   E   P   D   F   L   A   K   G
AGA CCT GGA TAC GGT GAG GCA TCA GAA GAG GAT AAA TTT CCG GCG AGA AGT   +1680
 R   P   G   Y   G   E   A   S   E   E   D   K   F   P   A   R   S
GAT GAT GTG GAA GTA GAG ACT GAG CTG GGA AGA GAC CCA AAG ACG GAG ACT   +1731
 D   D   V   E   V   E   T   E   L   G   R   D   P   K   T   E   T
CTT GAT CAA TTC TCA CCG GAA CTT TCT CAT CCT AAA GAA AGA GAT GAG TTT   +1782
 L   D   Q   F   S   P   E   L   S   H   P   K   E   R   D   E   F
AAG GAG TCC AGA GAT GAT TTT GAG GAG ACG AGA GAT GAG AAA ACA GAG GAG   +1833
 K   E   S   R   D   D   F   E   E   T   R   D   E   K   T   E   E
CCA AAA CAG AGC ACT TAC ACA GAG AAG TTT GCT TCA ATG CTA GGT TAC TCC   +1884
 P   K   Q   S   T   Y   T   E   K   F   A   S   M   L   G   Y   S
GGA GAA ATT CCG GTG GGA GAT CAA ACT CAA GTG GCG GGA ACT GTT GAT GAG   +1935
 G   E   I   P   V   G   D   Q   T   Q   V   A   G   T   V   D   E
AGG TTG ACT CCG GTC AAT GAG AAG GAT CAA GAA ACA GAG TCT GCC GTG ACG   +1986
 K   L   T   P   V   N   E   K   D   Q   E   T   E   S   A   V   T
ACG AAG TTA CCT ATC TCC GGA GGT GGA AGT GGA GTA GAG GAG CAA CGA GGG   +2037
 T   K   L   P   I   S   G   G   G   S   G   V   E   E   Q   R   G
GAA GAT AAA AGT GTG TCG GGT AGA GAT TAT GTG GCG GAG AAA CTG ACA ACT   +2088
 E   D   K   S   V   S   G   R   D   Y   V   A   E   K   L   T   T
GAA GAA GAA GAC AAA GCC TTT TCT GAT ATG GTT GCC GAG AAA CTT CAG ATT   +2139
 E   E   E   D   K   A   F   S   D   M   V   A   E   K   L   Q   I
```

Figure 3.1 cont

GGA
G
TCT
S
AAA
K
GCC
A
AAA
K
TC
S
CC
P
tt

ca

tg

ct

```
GGA GGA GAA GAA GAG AAG AAG GAA ACG ACG ACA AAG GAA GTG GAG AAG ATC  +2190
 G   G   E   E   E   K   K   E   T   T   T   K   E   V   E   K   I
TCT ACC GAG AAG GCA GCA TCG GAG GAG GGT GAG GCG GTG GAA GAG GAA GTG  +2241
 S   T   E   K   A   A   S   E   E   G   E   A   V   E   E   E   V
AAA GGA GGA GGA GGA ATG GTT GGG AGG ATT AAA GGA TGG TTC GGT GGT GGT  +2292
 K   G   G   G   G   M   V   G   R   I   K   G   W   F   G   G   G
GCG ACT GAT GAG GTG AAG CCA GAA TCG CCA CAT TCT GTT GAA GAG GCT CCA  +2343
 A   T   D   E   V   K   P   E   S   P   H   S   V   E   E   A   P
AAA TCA TCT GGC TGG TTT GGT GGT GGT GCG ACG GAG GAG GTG AAG CCA AAA  +2394
 K   S   S   G   W   F   G   G   G   A   T   E   E   V   K   P   K
TCG CCT CAT TCC GTT GAA GAG TCT CCA CAA TCA CTT GGC TCC ACT GTT GTT  +2445
 S   P   H   S   V   E   E   S   P   Q   S   L   G   S   T   V   V
CCG GTG CAG AAG GAG CTT taagaatatgagaactgagattttcaagtttcactttggatgt  +2506
 P   V   Q   K   E   L   *
ttatgtgtgttttgtttgacgtctttgatgtattatggtataattccttgtttgtgtgaaaaagga  +2573
                                |-> poly A
catttggttaataaattgttcggctttggattaagaagttcctccataccagctactaggtctaaag  +2640

tgggtaaaatcattggatttattcccttcaaagttcttagaattattcacaggatttacattatgag  +2707

ctagtagt                                                             +2715
```

Figure 3.1 cont

Figure 3.2 A ^{32}P-end-labeled 20 bp primer that hybridized to the sense strand of the *cor78* gene from bp +39 to +59 was mixed with 100 ug of total RNA from cold treated (lane 1) or control plants (lane 2), and extended by MlV reverse transcriptase. The product was run out on denaturing poly acrylamide gel. The same primer was used in a sequencing reaction of the *cor78* genomic clone for use as a size marker, and the sequence corresponding to the sense strand is denoted to the left of the figure.

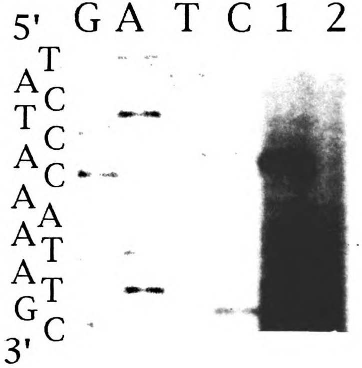


Figure 3.2

nontranslated leader sequence. The start of transcription, as determined by primer extension (Figure 3.2), was located 27 bps 3' to a putative TATA box. The sequences surrounding the start site, CTCACAA, loosely fit the consensus for plant transcription start sites, CTCATCA (Joshi, 1987). Earlier hybrid select and hybrid arrest experiments indicated that the partial cDNA for *cor78* corresponded to the 160kD boiling soluble cold-regulated protein observed in previous *in vitro* translations and *in vivo* labeling experiments of mRNA and proteins (respectively) from cold-treated *Arabidopsis* (Lin *et al.*, 1990; Gilmour *et al.*, 1988). However, sequencing of the near full length *cor78* cDNA indicated that this gene should encode a protein of 711 amino acids with a molecular weight of 77.9 kD. In order to determine if the cloned gene encoded the observed COR78 protein, RNA from the near full length cDNA was transcribed *in vitro*, translated, and analyzed by SDS-PAGE (Figure 3.3). The results indicated that this cDNA does indeed encode a boiling soluble protein that runs at 160kD (Lin *et al.*, 1990).

Analysis of the predicted structure of the COR78 suggested that the protein it encoded would be very hydrophilic (Figure 3.4) and acidic (pI=4.25). Both of these predictions are in agreement with the observations that this protein is boiling soluble and acidic as determined experimentally by 2-D SDS-PAGE analysis (Gilmour *et al.*, 1988). A number of repeated amino acid sequences were evident in COR78 (Figure 3.1). A search of the GenBank/EMBL (modified) data base (release70.0/29.0) showed no significant homology with any known proteins. Also, homology plots were performed to compare the amino acid sequence of COR78 with other known COR proteins, and with all of the known molecular chaperones and many plant heat shock genes. Again, no significant homologies were found. Finally, the repeated amino acid sequences within the COR78 protein were checked against the PROSITE data base

Figure 3.3 *In vitro* transcription and translation of pBM1. 10 ul of water as a negative control (designated N), 0.1 ug of RNA from *in vitro* transcribed pBM1 (designated I), 10 ug of total RNA from *A. thaliana*, ecotype Landsberg erecta that had been cold treated at 5°C overnight (designated C), or 10 ug of total RNA from warm controls (designated W) was added to a rabbit reticulocyte lysate in the presence of 35S methionine and in vitro translated. Each reaction was boiled, centrifuged, and the supernatant was run out on an SDS-PAGE gel.

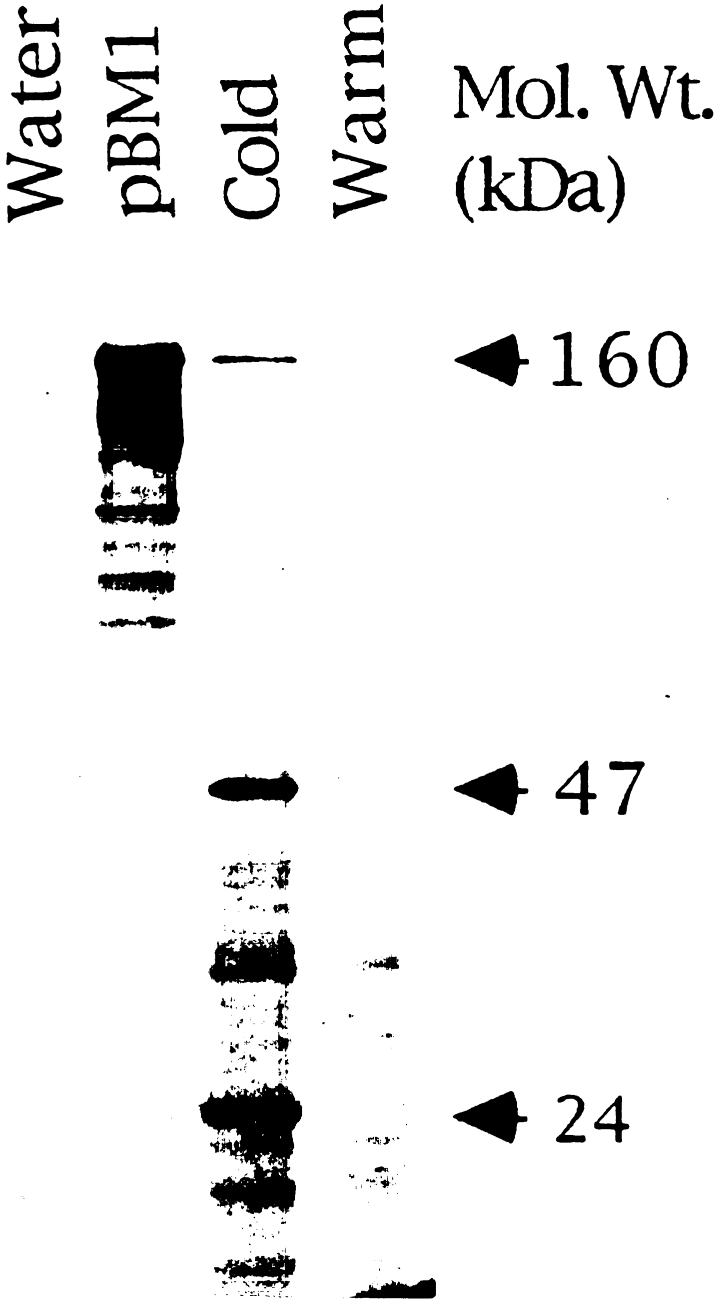


Figure 3.3

Figure 3.4 Hydrophilicity plot of *cor78*. Primary sequence data from the near full length cDNA clone (pBM1) was used to predict the protein structure of COR78. This information was used to generate a hydrophilicity plot of the predicted *cor78* protein utilizing the MACVECTOR 3.5 program. Data output was used directly for this figure.

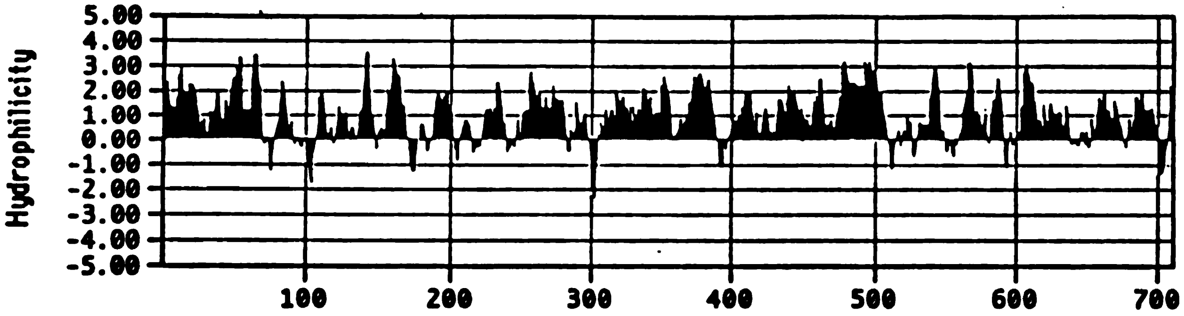

Hydrophilicity
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
-5.00
100
200
300
400
500
600
700

Figure 3.4

(release 8.0). No similarity to any part of any known protein was found.

The promoter of *cor78* is regulated by cold, drought, and ABA.

As an initial step in defining sequences that are important for the regulation of the *cor78* gene, a construct was prepared such that the promoter region from base pair -808 through +5 (construct 78P) was ligated into pBI101. Another construct containing the *cor78* promoter plus a portion of the *cor78* coding region from base pair -808 to +250 (construct 78PI, which includes the first intron) was ligated in frame to the GUS gene from pBI101.2. These constructs were then used to transform *Arabidopsis* (Figure 3.5A).

Initial characterization by northern analysis indicated that the endogenous *cor78* gene was induced by cold, ABA, and drought (Hajela *et al.*, 1990). Also, primer extension analysis indicated that when the *cor78* gene was induced with ABA, the transcription initiation site was the same as that of cold-induced transcription (data not shown). A transgenic line containing the *78Pgus* construct was grown on GM plates and either placed at 2^{o}C overnight, sprayed with 100 uM ABA for four hours, drought stressed by leaving the plate lid open overnight, or left untreated. RNA was extracted, blotted, and probed with a ^{32}P labeled, gel purified fragment comprising the GUS gene (Figure 3.5B). The GUS message accumulated 10-30 fold over the controls. In support of these data, the *78PIgus* construct also gave rise to a cold-regulated GUS message, but the GUS gene expressed from the CaMV promoter in pBI121 is not cold-regulated (Figure 3.5C). This experiment demonstrates that the *cor78* promoter appears to be provide sufficient regulation to account for the observed accumulation of the *cor78* message in cold, drought and ABA treated plants.

Figure 3.5A *A. thaliana* ecotype RLD were transformed with the following plasmids: pBI121, which has the GUS gene under the control of the CaMV 35S promoter (Jefferson *et al.*, 1988), pDH78P (construct 78Pgus), which contains a potion of the *cor78* gene from bp -808 to bp +5 ligated in front of the GUS gene in the plasmid pBI101.1 (Jefferson *et al.*, 1988), and pDH78PI (construct 78PIgus), which contains a portion of the *cor78* genomic clone from bp -808 to bp +250 (containing the first intron of the *cor78* gene) ligated in frame with the GUS gene from the plasmid pBI101.2 (Jefferson *et al.*, 1988).

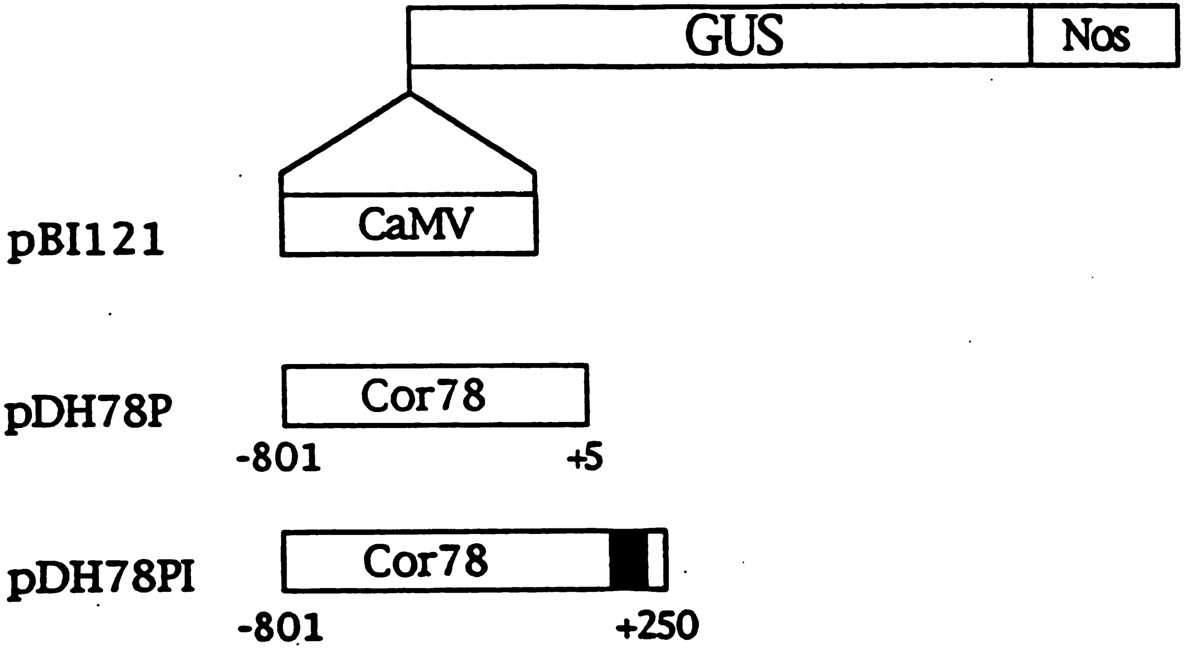


Figure 3.5A

Figure 3.5B Northern analysis of plants carrying the 78Pgus construct after drought, cold, and ABA treatment. A representative transgenic line of *Arabidopsis* plants that had been transformed with pDH78P were grown in petri dishes and drought stressed (D), left as warm controls (W), placed at 5^{0}C overnight (C), or sprayed with 100 uM ABA (A) prior to harvesting. RNA was extracted, and 10 ug of total RNA was run on a denaturing formaldehyde agarose gel for northern analysis. The probes were either ^{32}P labeled GUS gene from pBI121, or a ^{32}P labeled portion of the *cor78* gene from bp -808 to bp +250.

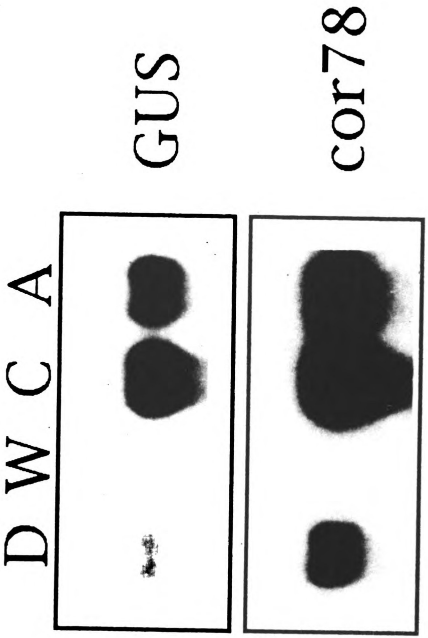


Figure 3.5B

Figure 3.5C Northern analysis of transgenic plants carrying promoter sequences of *cor78* driving GUS gene expression in the warm and cold. Representative transgenic lines of three week old *Arabidopsis*, transformed with either pBI121 (designated 35S), pDH78P (designated 78P2), or pDH78PI (designated 78PI3), were placed at 5°C (C) or left at 22°C (W). RNA was extracted, and 10ug of total RNA was run on a denaturing formaldehyde agarose gel for northern analysis. The probes were ^{32}P labeled GUS gene from pBI121, or a ^{32}P labeled portion of the *cor78* gene from bp -808 to bp +250.

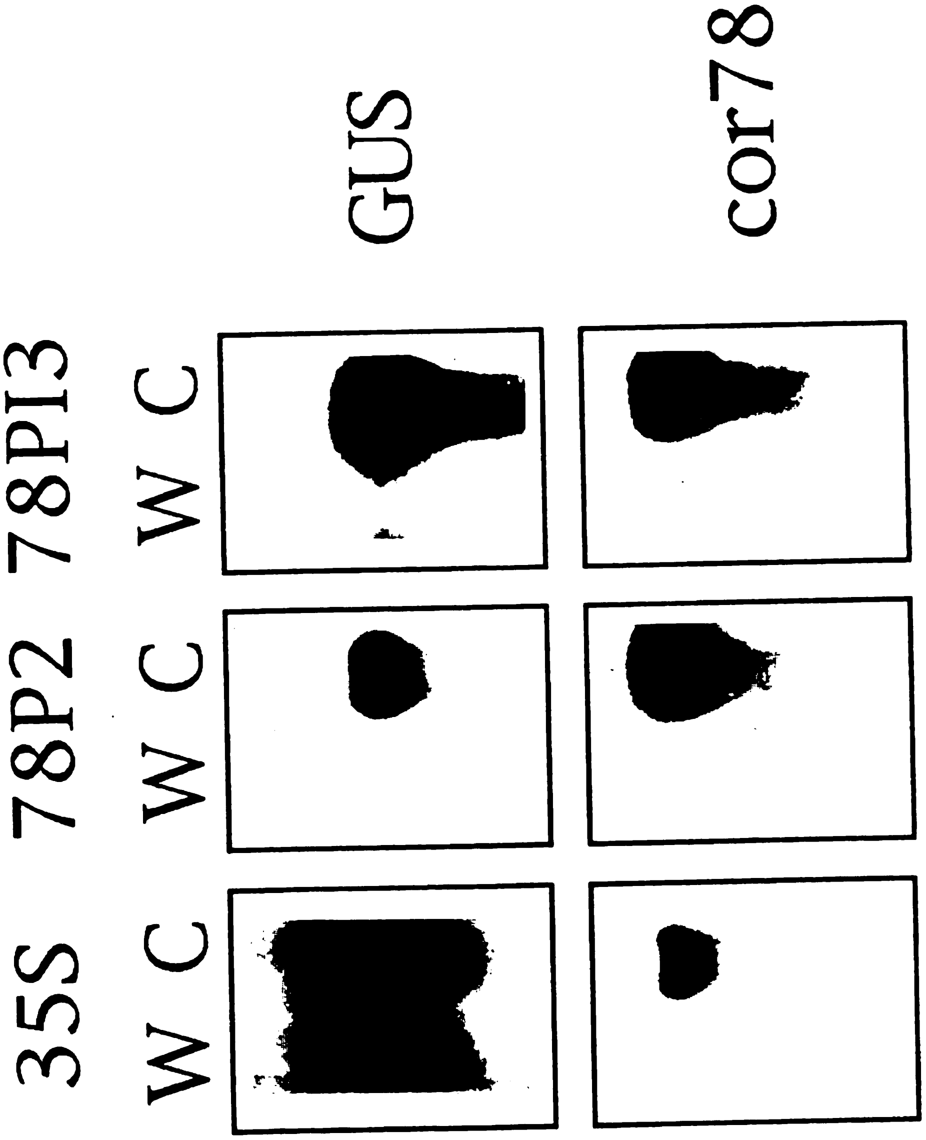


Figure 3.5C

Transcribed sequences do not impart cold-regulated expression to the *cor78* gene.

In order to determin whether that the transcribed region of the *cor78* gene contained sequences important for cold regulation, constructs containing portions of pHH28 were placed between the GUS gene from plasmid pBI131 (Jefferson *et al.*, 1987) and the nopaline synthase terminator (constructs *gus81C* and *gus410C*) (Figure 3.6A). Also, the entire transcribed region of the *cor78* gene, driven by the CaMV 35S promoter from pBI121 was prepared (construct *35Scor78*). These constructs were transformed into *Arabidopsis* (Figure 3.6B). Transformed lines were isolated and subjected to an overnight cold treatment along with a non-transformed control, and RNA was then isolated from these plants. Northern analysis on plants containing portions of the cDNA on the 3' end of GUS indicated that those constructs were incapable of imparting cold regulation to the reporter gene (Figure 3.6C). RNase protection assays were used to determine if message from construct containing the entire transcribed portion of *cor78* accumulated preferentially in the cold. The probe used for this experiment was a ^{32}P labeled, *in vitro* transcribed fragment from the genomic clone (homologous to the sense strand from bp -113 to +250). 117 bps of this probe is protected by the endogenous *cor78* transcript from the start of transcription to the 5' end of the first intron, and to the corresponding region of the *35Scor78* construct transcript plus the additional 11 bp of 5' sequences contained in this construct.

The results of the RNase protection assays (Figure 3.6D) show that in the cold-treated plants carrying the *35Scor78* construct, the message from both the endogenous *cor78* message and the *35Scor78* construct protect specific fragments. In the warm grown controls however, only the *35Scor78* transcript hybridizes to the probe. Unlike

Figure 3.6A *Arabidopsis* plants were transformed with the following plasmids: pBI131 (Jefferson *et al.*, 1989.) which has a GUS gene driven by the Small subunit of Rubisco followed by the nopaline synthase (Nos) terminator. pBI131 in which a portion of the 3' untranslated region of the *cor78* gene from the stop codon at bp +2463 up to, but not including, the putative poly A signal at bp +2582 ligated between the GUS gene and the Nos terminator (designated 81C). pBI131 in which a portion of the transcribed region of the *cor78* gene from bp +1510 to bp +2580 (again close to, but not including the putative poly A signal) ligated between the GUS gene and the Nos terminator (designated 410C). The poly A signal was left off in these constructs in order to prevent any interference with the poly A signal from the nopaline synthase terminator.

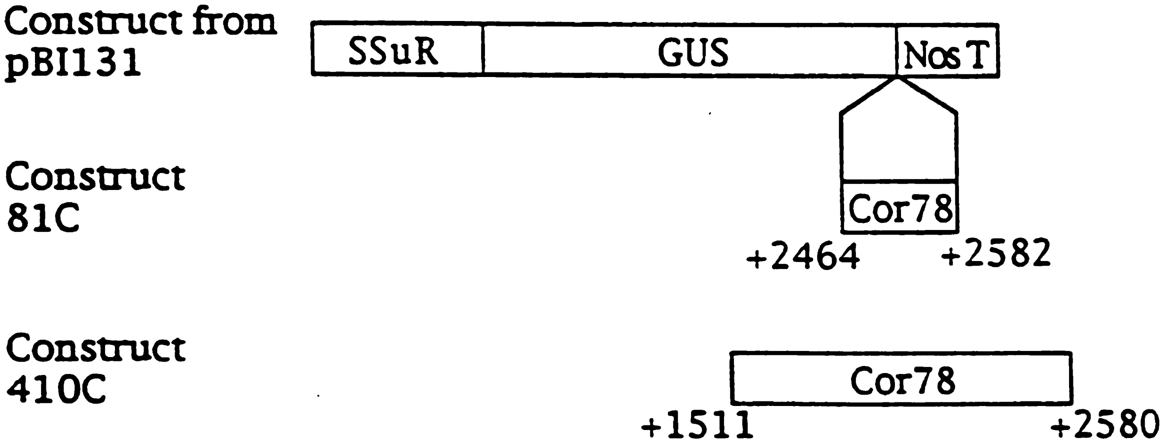

Construct from pBI131
SSuR
GUS
Nos T
Construct 81C
Cor78
+2464
+2582
Construct 410C
Cor78
+1511
+2580

Figure 3.6A

Figure 3.6B The construct *35Scor78* in the plasmid pDH78T contains a portion of the *cor78* genomic clone from bp -11 to bp +3000 placed under the control of the CaMV 35S promoter from plasmid pBI121. The insert shows the additional sequences (9 bps from the CaMV 35S gene (italicized), 11 bps from the pBI101 polylinker that was left after the construction of pBI121 including the XbaI site, and the 11 bps of *cor78* promoter sequences (underlined)) that would be transcribed as part of the 5'UTR from the *35Scor78* construct. The transcription of the addition of the 11bps from the *cor78* promoter makes it possible to distinguish between the endogenous *cor78* transcript and the transcript of *35Scor78* construct by using an RNase protection assay with the probe described below.

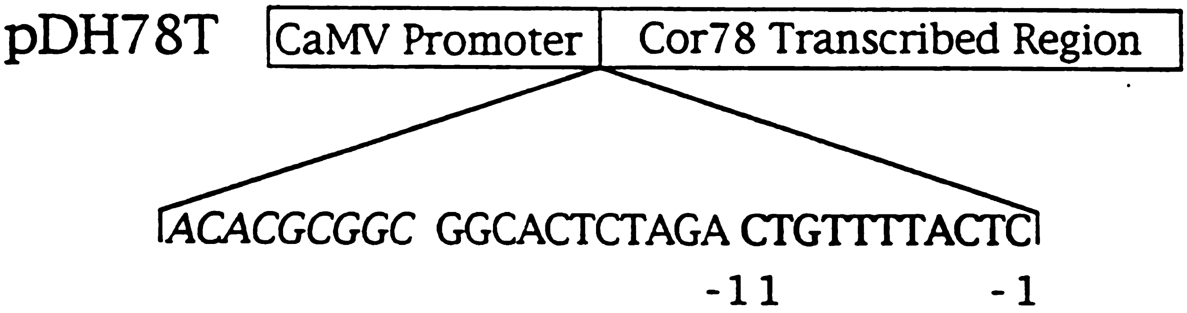

pDH78T
CaMV Promoter
Cor78 Transcribed Region
ACACGCGGC GGCACTCTAGA CTGTTTTACTC
-11
-1

Figure 3.6B

Figure 3.6C *Arabidopsis thaliana* ecotype RLD were transformed with pBI131 (lanes 1 and 2), pDH81C (lanes 3 and 4), and pDH410C (lanes 5 and 6) and either cold treated (lanes 1, 3, and 5) or left as untreated controls (lanes 2, 4, and 6). 10 ug of total RNA from each was run out on a denaturing formaldehyde agarose gel and subjected to northern analysis. The probes were ^{32}P-labeled gel isolated insert of either the GUS gene from pBI121, or a fragment of the *cor78* gene from -809 to +250.

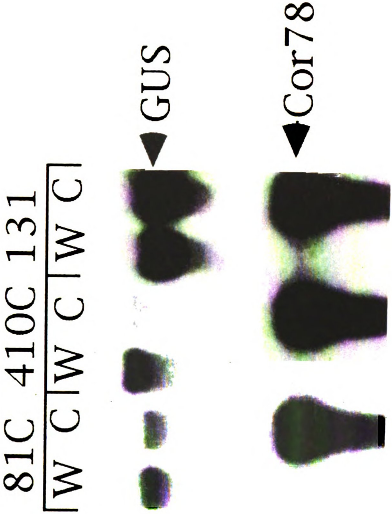


Figure 3.6C

Figure 3.6D RNase protection assay for detecting *35Scor78* transcripts from warm or cold treated transgenic and non-transgenic plants. *A. thaliana*, ecotype RLD, left as a non-transformed control (RLD), or a representative line which had been transformed with the plasmid pDH78T (78T2) were placed at 5^{o}C overnight (C) or left at 22^{o}C (W). Plants were harvested and RNA was extracted. 10 ug of total RNA was hybridized to a ^{32}P-labeled, *in vitro* transcribed RNA complimentary to the sense strand of the genomic clone from bp -113 to bp +250. The hybridization reaction was then treated with RNases A and T1 and run out on a 8% polyacrylamide sequencing gel.

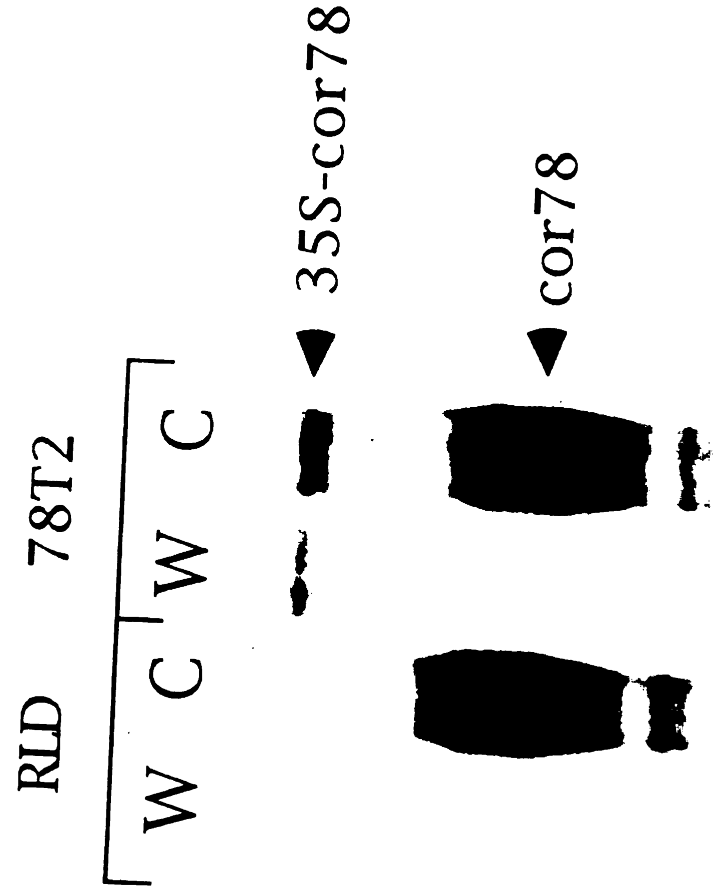


Figure 3.7D

the previous nuclear runon results, the data from these experiments suggest that transcribed sequences are not sufficient for imparting cold regulation to the *cor78* gene. It is possible however, that the additional 31bps (9bps from the CaMV promoter, 11bps from the polylinker of pBI101, and 11bps from the 5' nontranscribed region of *cor78*) interfered with the post-transcriptional control sequence(s).

Tissue specificity of the *cor78* promoter

Northern blot analysis indicated that *cor78* should be expressed in roots, stems, and leaves (see Figure A.9). To further define where in the plant the *cor78* promoter functions, plate grown (Figures 3.8A-C) or pot grown (Figures 3.8D-X) plants containing the *78PIgus* construct (which produced greater GUS activity than any of the *77Pgus* containing lines) were stained for GUS activity after a cold treatment (Figures 3.7A,D,G,J,M,N,O and W). Plants containing the 35S/gus construct were used as a positive control (Figures 3.7C,F,I,L,T and U). Staining for GUS in control plants indicated that GUS was not present or was present at very low levels in most of the tissues examined (Figures 3.B, E,H,K,P,Q,R,S,V and X). One exception to this was the trichomes in which there appeared to be significant levels of GUS activity (Figures 3.7S, and X). The GUS activity in the trichomes is most probably the result of a localized drought response since the same plant, when sprayed with water and covered in plastic wrap, lost GUS activity in the trichomes within 24 hours (Figure 3.7V). The same plant did demonstrate significant GUS activity in both the trichomes and other leaf cells after drought stress (Figure 3.7W) In cold-treated plants, the stems, leaves, sepals, and the filament portion of the stamen had high levels of GUS activity (Figure 3.7A,G,J and M). Low levels of activity were seen in petals, and the base and tips of

Figure 3.7A The tissue specificity of GUS gene expression from the *cor78* promoter was examined. Plants containing the *cor78/gus* construct (Columns 1 and 2) or plants containing the *35S/gus* construct (Column 3) were cold treated (Column 1) or left untreated (Columns 2 and 3). Plants were then stained for GUS activity (see Materials and Methods), and photographed under dark field illumination. Row 1 shows two week old plate grown plants. Row 2-4 show selected parts of four week old pot grown plants.

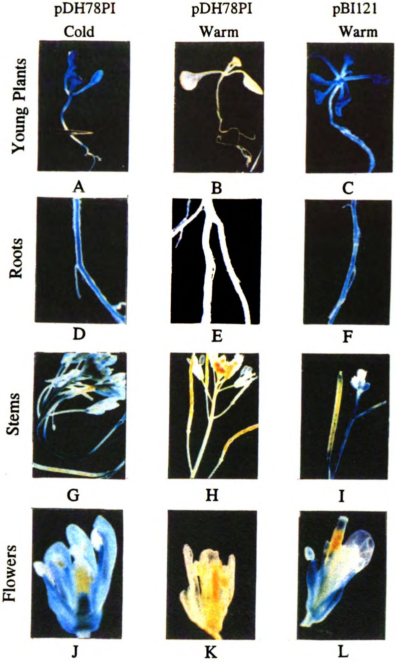

pDH78PI
Cold
pDH78PI
Warm
pBI121
Warm
Young Plants
A
B
C
Roots
D
E
F
Stems
G
H
I
Flowers
J
K
L

Figure 3.7

Figure 3.7 The tissue specificity of GUS gene expression from the *cor78* promoter was examined. Plants containing the *cor78/gus* construct or plants containing the *35S/gus* construct were cold stressed (cold), drought stressed (drought), sprayed with water and covered with plastic wrap for 24 hours (humid), or left untreated (warm). Plants were then stained for GUS activity (see Materials and Methods), and photographed under dark field illumination.

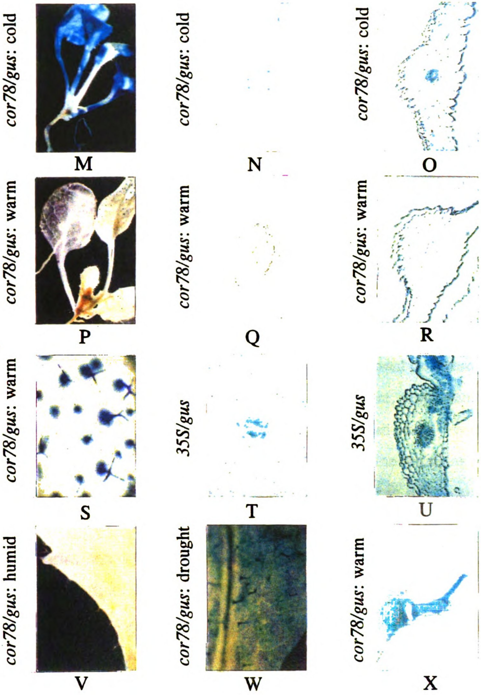


Figure 3.7 cont

the siliques. No GUS activity was observed in the anthers, pistils, or seeds. The lack of staining in the body of the siliques appears to be the result of poor infiltration of the GUS staining solution since a similar staining pattern was observed for plants transformed with pBI121. Also, siliques that were sliced with a razor blade turned blue upon staining (data not shown). The roots showed staining in the vascular tissue and pith, but little activity in the epidermis. Again, this same staining pattern was observed with plants containing the 35S/gus construct, and therefore the lack of staining probably does not reflect tissue specificity of GUS expression in response to cold.

Temporal regulation of the *cor78* promoter

In order to gain insight into the signal transduction pathway(s) involved in the induction of the *cor78* gene, it was of interest to study the time course of accumulation and loss of the endogenous *cor78* transcript as the plants were moved from room temperature to 2°C and then back to room temperature. Therefore, plants were grown on petri plates, each plate representing a sample time point, and all plates were placed in the cold with samples being harvested for up to three hours. The remaining plates were then placed back at room temperature and samples taken for an additional three hours.

From earlier results (Hajela *et al.*, 1990), it was observed that *cor78* mRNA accumulated to approximately one half its maximal levels within 4 hours. Thus it was assumed that the *cor78* gene would be activated and mRNA would begin to accumulate rather quickly upon a cold treatment. To follow the induction of this gene in the cold over times less than 4 hours, plate grown plants were placed in the cold and plants from a single plate were harvested every hour. RNA from was extracted and subjected to northern analysis. Surprisingly, it was found (Figure 3.8A) that *cor78* mRNA did not

Figure 3.8A A representative northern blot analysis of a time course of the induction and loss of the *cor78* message during and after a three hour cold treatment. Plants were grown on petri dishes and then placed at 5oC for three hours. Plants were harvested at 60, 120, 150, and 180 minutes after the begining of the cold treatment. Plants were then removed from the cold and samples were harvested every 15 minutes for the first hour and then at 90 and 120 minutes. The temperature of the agar surface on the plates were measured with a thermo-couple and are noted under each band.

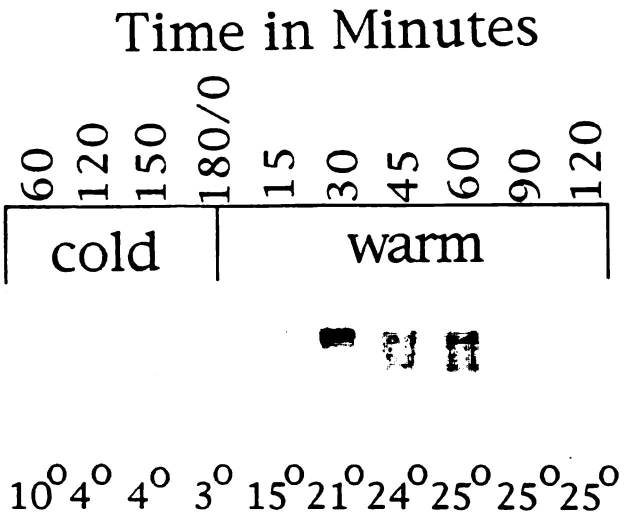

Time in Minutes
60
120
150
180/0
15
30
45
60
90
120
cold
warm
10°
4°
4°
3°
15°
21°
24°
25°
25°
25°

Figure 3.8A

Figure 3.8B Northern analysis of the kinetics of *cor78* expression. Plate grown *Arabidopsis* plants were placed at $3^{\circ}C$ for 3 hours and then moved back to room temperature. Plants were harvested every fifteen minutes from 0 minutes to 60 minutes and then every half hour for the last two time points. The surface temperature (in degrees C) of the agar from each experiment was measured with a thermocouple and is denoted above each data point across the upper X axis. RNA was then harvested from each time point, and run out on a gel for northern analysis. Gels were blotted and probed with ^{32}P-labeled *cor78* clone. Hybridization was quantified on a B-scope 3000. The counts were normalized as a percentage of the maximum counts in the hottest lane. The log of the average percent accumulation from two to three separate experiments was then plotted over time.

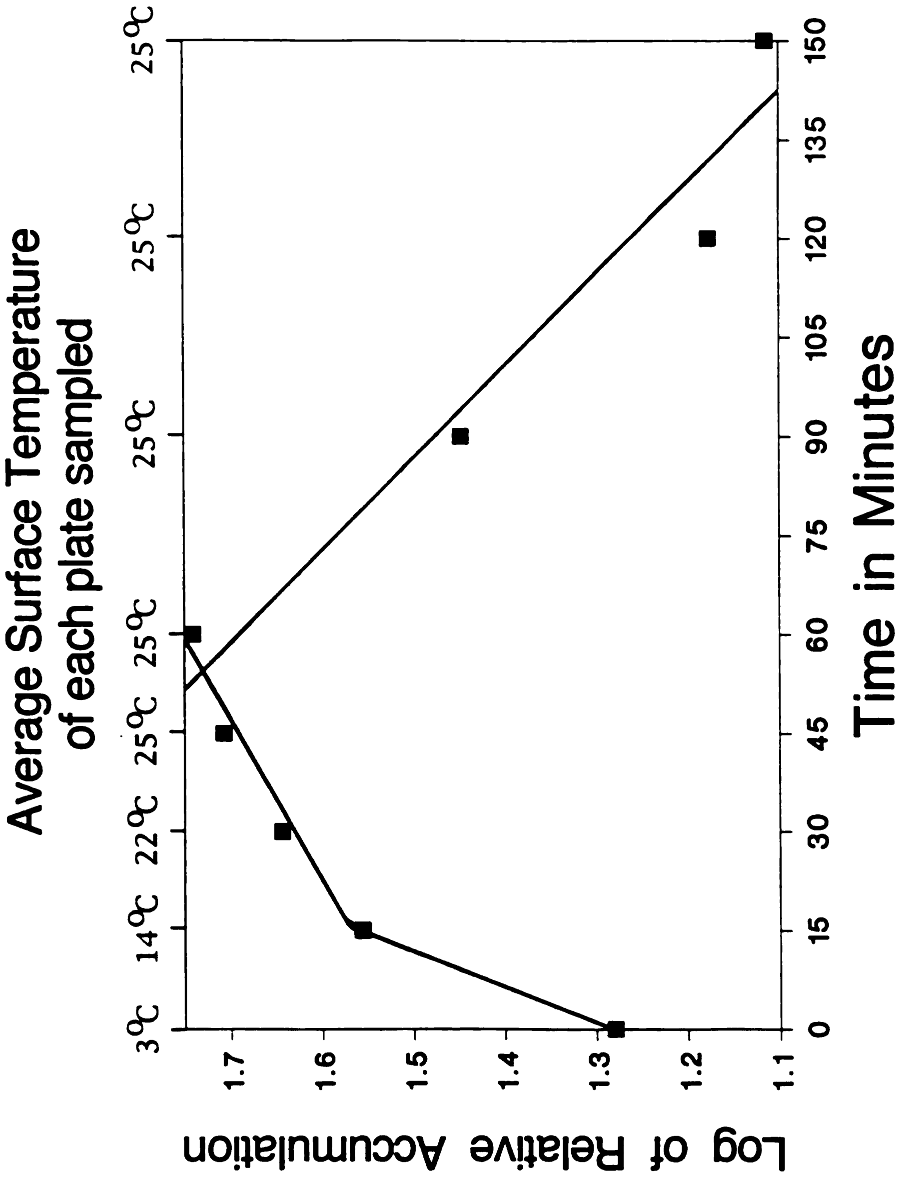

Average Surface Temperature
of each plate sampled
3°C
14°C
22°C
25°C
25°C
25°C
25°C
25°C
Log of Relative Accumulation
1.7
1.6
1.5
1.4
1.3
1.2
1.1
0
15
30
45
60
75
90
105
120
135
150
Time in Minutes

Figure 3.8B

Figure 3.8C Northern analysis of the kinetics of *cor78* expression. Plate grown *Arabidopsis* plants were placed at 3^{o}C overnight and then moved back to room temperature. Plants were harvested every fifteen minutes from 0 minutes to 60 minutes and then every half hour for the last two time points. The surface temperature (in degrees C) of the agar from each experiment was measured with a thermocouple and is denoted above each data point across the upper X axis. RNA was then harvested from each time point, and run out on a gel for northern analysis. Gels were blotted and probed with the *cor78* clone. Hybridization was quantified on a B-scope 3000. The counts were normalized as a percentage of the maximum counts in the hottest lane. The average of the log of the percent accumulation from two to three separate experiments was then plotted over time.

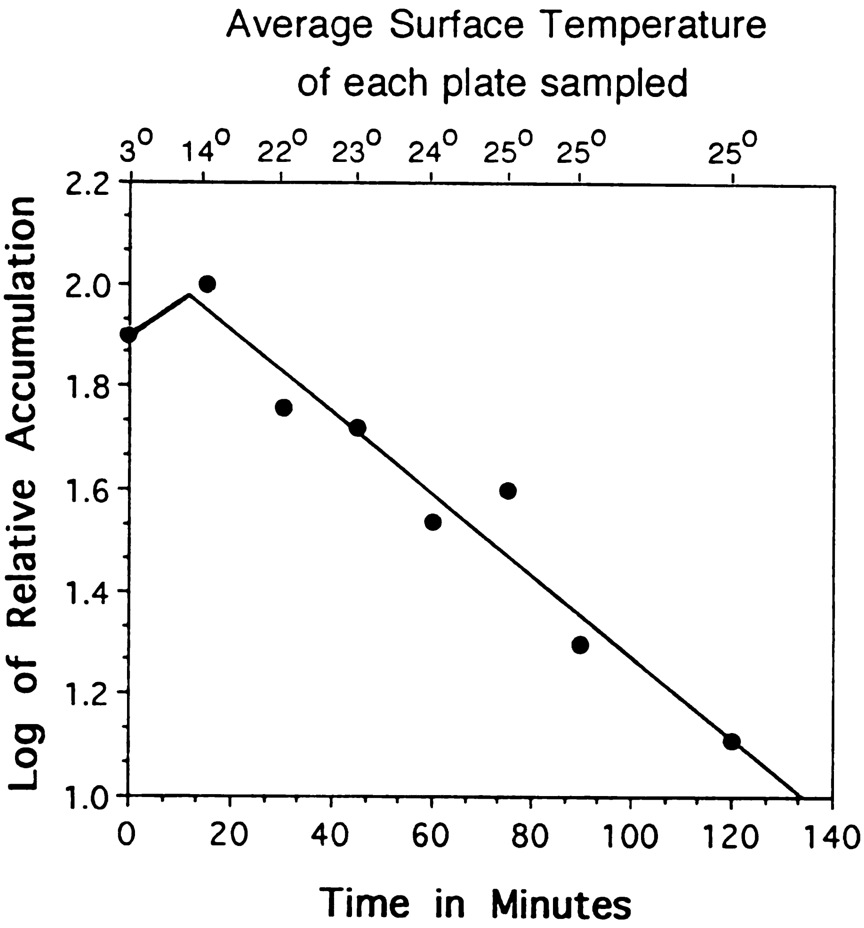

Average Surface Temperature
of each plate sampled
3°
14°
22°
23°
24°
25°
25°
25°
Log of Relative Accumulation
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0
20
40
60
80
100
120
140
Time in Minutes

Figure 3.8C

accumulate to detectable levels for nearly two hours after the plants had reached 10^oC (a temperature previously determined to be capable of inducing *cor* gene accumulation (Thomashow *et al.*, 1990). What was perhaps more surprising were the findings that the level of *cor78* mRNA continued to rise for 45 to 60 minutes after the plants had been returned to normal growing temperatures (Figures 3.8A and B). Interestingly, the lag time observed for the loss of message upon the return of the plants to normal growing temperatures after three hours in the cold was not observed when the plants were left in the cold overnight (Figure 3.8C). Finally, once the loss of the *cor78* message had begun to occur, it consistently demonstrated a half-life of about 35 minutes in nearly all of the samples tested.

DISCUSSION

Analysis of the transcribed region of the *cor78* gene

In the 5' untranslated leader of *cor78*, there are two possible ATG codons that may initiate translation (see Figure 1). The first ATG following the start of transcription lacks most of the consensus sequences (AACA<u>ATG</u>GC) normally found around active translational initiation sites in plants (Joshi, 1987). Also, translation initiation from the first ATG would not lead to a long open reading frame. The second ATG however provides a much better match to the consensus sequence. Therefore, we believe that it is this second ATG that actually is used to initiate translation.

Assuming that the second ATG is the true initiation codon, the protein encoded by *cor78* is predicted to be 77.9 kD in size. However, when RNA from the full length cDNA was *in vitro* transcribed and translated, SDS-PAGE analysis indicated that it ran as a 160 kD polypeptide (as does the endogenous *cor78* gene product). Detergents, high temperature, guanidium sulfate, urea, and formamide, were used in attempts to break apart any possible dimer formed by this protein, but in all cases, the *in vitro* translation product ran as a 160kD protein (Gilmour, unpublished results). One possible explanation for why COR78 runs anomalously through an SDS-PAGE gel is that the hydrophilic nature of this protein reduces its affinity for SDS, and thereby resulting in a protein that migrates more slowly through the electrophoretic field (Nelson, 1971; Panyim and Chalkley, 1971). The hydrophilic or acidic nature of COR78 has led to speculation concerning its function. It is possible that the highly charged nature of COR78 may allow it to function as a compatible solute (Carpenter and Crowe, 1988), and thus possibly protect other cellular components during freezing and dehydration stress.

Sequences within the *cor78* promoter may play a role in ABA and drought regulation of this gene

Initial characterization of the endogenous *cor78* gene indicated that, in addition to cold, it was induced by both ABA and drought (Hajela *et al.*, 1990). Northern analysis indicated that the *cor78* promoter, by itself, is capable of conferring ABA and drought regulation to the GUS reporter gene. Interestingly, sequence data indicated the presence of a G box like element (TACGTG) at position -66 (26 bps 5' to the presumed TATA box). This is of great interest, since G box like elements have been implicated in ABA responsiveness in a number of plant promoters (Lam and Chua, 1991; Mundy *et al.*, 1990; Guiltinan *et al.*, 1990).

G-box like elements have been found in the same proximity (25-30bp 5') to the TATA box in the promoters of two other *cor* genes from *Arabidopsis* (Horvath, unpublished results) and in a *cor* gene from *Brassica napus* (Singh *et al.*, personal communication). Interestingly, the G-box from the *cor78* promoter has been shown to act as a binding site for the DNA binding factor GBF1 (Schindler *et al.*, 1992). The location of the G boxes in these *cor* genes may be of significance, since they are much closer to the TATA box than has been observed with the G boxes implicated in ABA responsiveness from other plants (normally found between 189-270 in other species) (Mundy *et al.*, 1990). It is not yet known if the presence and/or position of the G box in the *cor78* gene is of significance for cold regulation. However, preliminary data has indicated that the region from -240 through the TATA box (which includes both the G Box like element and the direct repeat (described below) is essential for drought activated regulation from this promoter in transgenic tobacco plants (Kazuko *et al.*, unpublished results).

Another interesting sequence that was found in the *cor78* promoter was a 21 bp direct repeat at bp 120 and bp 210. Within this repeat is a 5 bp sequence (CCGAC) that has been found in all of the cold-regulated promoters from both *Arabidopsis* and *Brassica napus* found to date (Table 3.1). Interestingly, a short (180 bp) fragment from the promoter of *cor15* was shown to confer cold-regulation to the CaMV 35s-90 minimal promoter in preliminary transient expression studies (McLarney, unpublished results). The only conserved sequence within the 180 bp fragment of *cor15* was the 5 bp sequence observed in the repeat of *cor78*.

Expression of the *cor78* constructs and implications of the nuclear runon transcription assays

The results of previous nuclear runon experiments (Hajela *et al.*, 1990) suggested that the *cor78*, *cor47* and *cor6.6* were post-transcriptionally regulated. Thus one could expect that sequences important for their cold regulation would be located within the transcribed regions of these genes, and the promoter from cor78 would

Table 3.1

Location and Sequence of ICE-Box

Gene	Sequence	Position
Arabidopsis cor78 R1	ACCGACATCAGTT	-166
Arabidopsis cor78 R2	ACCGACATGAGTT	-225
Arabidopsis cor15a R1	GCCGACATACATT	-183
Arabidopsis cor15a R2	GCCGACCTGCTTT	-360
Arabidopsis cor15b	GCCGACCTCTTTT	- 196
Consensus	RCCGACMTNNTT	

Conserved sequence found in the promoter of several *cor* genes. Two copies of this element are found in *cor78* (R1 and R2 respectively). Two copies are found in *cor15a* (R1 and R2) and one copy in *cor15b*. The position of each element is listed relative to the known (*cor78* and *cor15a*) or putative (*cor15b*) transcriptional start sites of each gene. The consensus sequence for this "Induced by Cold Element" (ICE-Box), is shown.

be active constitutively. However, the results of the *cor78*/GUS fusion constructs appear to contradict this. For these two results to be compatible, it would have to be concluded that the sequences between +1 and +5 contain all the necessary information for cold regulation. In light of the data which demonstrated that the *cor78* transcribed region (containing the sequences from +1 to +5) was not cold-regulated when placed under the control of the constitutive CaMV 35S promoter (see Figure 3.7D), this possibility seems unlikely. Although it is possible that the 31 bps of additional sequences 5' to the normal transcription start site interfered with the regulation of this construct, the most likely interpretation of these data is that the sequences controlling cold regulation are not contained in the transcribed region of *cor78*. It was also possible (although unlikely) that there were sequences that caused the *cor78* gene to be constitutively expressed, but that these sequences were not included in the *cor78* promoter/GUS constructs. In order to test this possibility, nuclear runon assays were done on transgenic plants containing the *78P/gus* constructs (Data not shown). Preliminary results from these nuclear runon experiments show that the *cor78/gus* constructs are regulated in the same manner as the endogenous *cor78* gene. This then suggests that all of the information necessary for controlling the GUS reporter gene in the same manner as the endogenous *cor78* (i.e., making it also appear to be constitutively expressed in nuclear runon assays), are contained within the region between -808 to +5 of the *cor78* gene.

Since it appears that the promoter region of the *cor78* gene is responsible for its cold regulation, the nuclear runon results indicating that this gene is expressed constitutively may provide some interesting insight into how this gene is regulated. One possible explanation for the observed accumulation of *cor78* message in nuclei from control *Arabidopsis*, is that there is a poised polymerase present on the *cor78* promoter which is released during the isolation procedure. Such poised polymerases have been observed on the *Hsp70* promoter from *Drosophila* (Rougvie and Lis, 1988), and it has been demonstrated that certain treatments (such as high potassium concentrations or detergents), could release the polymerase and allow transcription *in vitro*. Although the nuclear isolation procedure for *Arabidopsis* would not expose the nuclear to either of these conditions, the possibility for the release of a polymerase still exists since the nuclear isolation procedures for *Arabidopsis* (which uses ether to dissolve the nuclear membrane) and *Drosophila* (which uses nonidet P40) are quite different.

Recent work on the *cor78* gene indicates that it may be part of a multi-gene family (Yamaguchi-Shinozaki and Shinozaki, 1992). Thus it is possible that one or more of the members of this family are constitutively transcribed, but others are inducible. Consequently, if I happened to clone one of the transcriptionally regulated copies of *cor78*, then the discrepancy between the nuclear runon results and the results from the construct data need not be mutually exclusive.

Another explanation for the observed nuclear runon results may be that some treatment during the nuclear isolation procedure activates the *cor78* promoter. Since it is known that drought stress can induce the *cor78* gene, it is possible that during the time when the *Arabidopsis* plants are being diced, the loss of turgor pressure caused by the physical disruption of the cell membranes was enough of a stimulus to cause the *cor78* promoter to be activated. In light of recent studies that have shown that the *cor78* gene is expressed at high enough levels to be detected by northern analysis in less than 20 minutes upon dehydration (Kazuko *et al.*, 1992), this appears to be the most likely explanation.

Implications of the data from the temporal expression of *cor78*.

The results from the time course studies on the accumulation and loss of the *cor78* mRNA demonstrated that there was a lag period of about 2 hours between the time the plants experienced inducing conditions (10^{O}C) and the time it took before *cor78* mRNA accumulated to detectable levels. Assuming that there is no significant effect on the general rate of transcription due to the cold *per se*, these data suggest that there is some complex or slow metabolic pathway involved in the induction process of *cor78* (thus negating the possibility of having a trans-acting factor that directly senses cold). If however, the cold does have a general effect on the rate of transcription, then it is possible that there is some trans-acting factor that directly senses the cold, and binds to the promoter once the inducing temperature is reached (thus activating the gene). However, the RNA polymerase may have to undergo some modification in order to adjust to transcription in the cold before reaching full activity. Therefore, the lag period prior to induction of *cor78* could be the result of the time it takes to modify the RNA polymerase rather than the time it takes to activate the *cor78* promoter. Although no short term measurements of transcription rates have been reported for plants undergoing a cold stress, such studies have been done in *E. coli*. In these experiments, it was determined that the rate of transcription did fall in bacteria undergoing a cold shock, but that the rates recovered to near normal levels within 15 minutes (Mackow and Chang, 1989). It should be however, relatively easy to determine if transcription from the *cor78* promoter is inhibited for any significant length of time by the cold *per se*. Work by Yamaguchi-Shinozaki and Shinozaki., (1992) has shown that drought stress is capable of inducing *cor78* in as little as 20 minutes. Therefore, one should be able to determine if the cold has a direct inhibitory effect on the accumulation of the *cor78* transcript by simultaneously drought stressing and cold stressing plants. It is noted here however, that such an experiment would be conclusive only if it gave positive results. If it turned out that the drought treatment was not capable of inducing *cor78* in the cold, no conclusion could be immediately drawn since the cold treatment could directly alter the effectiveness of the drought treatment (i.e. by slowing evaporation).

Another interesting finding from the time course experiments was the observed difference in the loss of *cor78* mRNA between plants that were left in the cold for three hours, and those that were left in the cold overnight. Plants left in the cold for three hours increased or at least maintained significant levels of *cor78* mRNA for up to 60 minutes after the plants were returned to room temperature. Since the plants had reached non-inducing temperatures within the first 15 minutes after were removed from the cold, the *cor78* promoter was apparently active for at least 45 minutes under non-inducing conditions. In contrast, the *cor78* message started to disappear after only 15 minutes when plants were left in the cold overnight prior to returning them to room temperature. These results suggest that there is some difference in the way the *cor78* gene is regulated after experiencing three hours of cold as opposed to 12 hours of cold.

There are two possible models that may explain these differences. One implies that the mechanisms involved in the initial induction of the gene are different than those that are involved in maintaining expression over longer periods of time. The other implies that there is more than one mechanism that is affecting *cor78* accumulation shortly after induction. It is possible that the processes involved in the induction of this gene (perhaps the buildup of a particular metabolite as the result of a cold sensitive enzyme) activate a signal transduction pathway. This could lead to a number of changes including the induction of a trans-acting factor that is critical for its own transcription and that of the *cor78* gene. In this scenario the lag in the loss of the *cor78* RNA in 3 hour cold-treated plants would be the result of the time it would take to reduce the amount of the critical metabolite back to non-inducing levels.

The second scenario would be that there is some trans-acting factor that is activated by cold temperatures and inactivated at warmer temperatures. However, during the initial stages of the cold stress, there are several other physiological events occurring, such as an increase in ABA levels, that can affect the induction of the *cor78* gene. Increases in ABA levels during the initial stages of cold acclimation have been documented in several systems (Daie and Campbell, 1981; Chen *et al,* 1983). Therefore, the lag in the loss of the *cor78* mRNA in 3 hour cold-treated plants could be the result of continued induction by ABA, rather than a carry-over of some mechanism that was specifically responsive to temperature *per se.*

Finally, the results from these time course studies demonstrate that the *cor78* message has an unusually short half-life (on the order of 35 minutes), and that the *cor78* promoter is essentially inactivated in less than 15 minutes after reaching noninducing conditions (at least in plants that were cold-treated overnight). These two observations together suggest that the expression of this gene very tightly controlled, and thus it may be deleterious for the plant to express it under optimal growing conditions. If this protein affects the way water interacts with important structures within the cell, such as membranes or critical enzymes, (as discussed in Chapter 1) then the COR78 protein may cause significant problems in unstressed plant cells.

The rapidity of the loss of the *cor78* message brings with it a number of other implications and potentially interesting side studies. If the *cor78* promoter is indeed turned off as rapidly and completely as it seems to be, it may make for a very useful expression system for studying the half lives of other RNAs. In the same vein, it should be of general interest to determine what sequences or structures, contained within the *cor78* message, are responsible for its apparent instability. In fact, the constructs DH81C, and DH410C may be useful in looking for such instability sequences. One of these constructs (pDH81C) carries just the 3'UTR of the *cor78* mRNA added to the 3'UTR of GUS. Given the relative AU richness of this sequence, (AU richness being generally associated with mRNA stability, Ross, 1989), it will be of interest to see if these sequences (in either orientation) alter the stability of the GUS message.

Literature Cited

Carpenter JF, Crowe JH (1988) The mechanism of cryoprotection of proteins by solutes. Cryobiology 25, 244-255

Chen HH, Li PH, Brenner ML (1983) Involvement of abscisic acid in potato cold acclimation. Plant Physiol. 71,362-365

Daie J, Campbell WF (1981) Response of tomato plants to stressful temperatures. Plant Physiol. 67, 26-69

De Block M, Debrouwer D (1992) *In-situ* enzyme histochemistry on plastic-embedded plant material. The development of an artifact-free -glucuronidase assay. The Plant Journal 2(2), 261-266

Gilmour SJ, Hajela RK, Thomashow MF (1988) Cold acclimation in *Arabidopsis thaliana*. Plant Physiol. 87, 745-7750

Gilmour SJ, Thomashow MF (1991) Cold acclimation and cold-regulated gene expression in ABA mutants of *Arabidopsis thaliana*. Plant Mol. Biol. 17, 1233-1240

Guiltinan MJ, Marcotte WR, Quatrano RS (1990) A plant leucine zipper protein that recognizes an abscisic acid response element. Science 250, 267-270

Hajela R, Horvath DP, Gilmour SJ, Thomashow MF (1990) Molecular cloning and expression of *cor* (cold-regulated) genes in *Arabidopsis thaliana*. Plant Physiol. 93, 1246-1252

Jefferson RA, Kavanaugh TA, Bevan MW (1987) GUS fusions: -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. The EMBO Journal 6, 3901-3907

Joshi CP (1987) An inspection of the domain between putative TATA box and translation start sites in 79 plant genes. Nucl. Acids Research 15, 6643-6653

Kasperska-Palacz A , Dlugokecka E, Breitenwald J, Wcislinska B (1977) Physiological mechanisms of frost tolerance: possible role of protein in plant adaptation to cold. Biol. Plant. 19, 10-17

Yamaguchi-Shinozaki K, Shinozaki K (1992) Characterization of the expression of a desiccation-responsive rd29 gene of *Arabidopsis thaliana* and analysis of its promoter in transgenic plants. Mol. Gen. Genet. in press.

Kyte, Doolittle (1982) A simple method for displaying the hydropathic nature of a protein. J. Mol. Biol. 15, 105-132

Laemmli UK (1977) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685

Lam E, Chua N-H (1991) Tetramer of a 21-base pair synthetic element confers seed expression and transcriptional enhancement in response to water stress and abscisic acid. J. Biol. Chem. 266, 17131-17135

Levitt J (1980) Responses of plants to environmental stress. Chilling, freezing, and high temperature stresses. Ed 2, Academic Press, New York

Lin CT, Guo W, Everson E, Thomashow M (1990) Cold acclimation in *Arabidopsis* and wheat. Plant Physiol. 94, 1087-1083

Lin CT, Thomashow (1992) DNA sequence analysis of a complimentary DNA for cold-regulated *Arabidopsis* gene *cor15* and characterization of the *COR15* polypeptide. Plant Physiol. 99,

Mackow ER, Chang FN (1989) Correlation between RNA synthesis and ppGpp content in *Escherichia coli* during temperature shifts. Mol. Gen. Genet. 192, 5-9

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Mundy J, Yamaguchi-Shinozaki K, Chua N-H (1990) Nuclear proteins bind conserved elements in abscisic acid-responsive promoter of a rice *rab* gene. PNAS 87, 1406-1410

Nelson CA (1971) The binding of detergents to proteins. J. Biol. Chem. 246, 3895-3901

Nordin K, Pekka H, Palva E (1991) Separate signal pathways regulate the expression of a low-temperature-induced gene in *Arabidopsis thaliana* (L.) Heynh. Plant Mol. Biol. 16 1061-1071

Panyim S, Chalkley R (1971) The molecular weights of vertebrate histones exploiting a modified sodium Dodecyl sulfate electrophoretic method. J. Biol. Chem. 246, 7557-7560

Rougvie AE, Lis JT (1988) The RNA polymerase II molecule at the 5' end of the uninduced *hsp70* genes of *D. melanogaster* is transcriptionally engaged. Cell 54, 795-804

Ross J (1989) The turnover of messenger RNA. Scientific American Apr. pp 48-55

Sakai A, Larcher W. (1987) Frost survival of plants; response and adaptation to freezing stress. Spring-Verlag, New York

Sambrook J, Fritsh EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Ed2. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Schindler U, Terzaghi W, Beckmann H, Kadesch T, Cashmore T (1992) DNA binding site preferences and transcriptional activation properties of the *Arabidopsis* transcription factor GBF1. The EMBO Journal 11, 1275-1289

Steponkus P, Uemura M, Balsmo R, Arvinte T, Lynch D (1988) Transformation of the cryobehavior of rye protoplasts by modification of the plasma membrane lipid composition. PNAS 85, 9026-9030

Thomashow M, (1990) Molecular genetics of cold acclimation in higher plants. In Scandalios JG, and Wright TRF, eds. Advances in genetics, Vol. 28, Genetic Responses to Environmental Stress. Academic Press, New York

Thomashow M, Gilmour S, Hajela R, Horvath D, Lin C, Guo W (1990) Studies on cold acclimation in *Arabidosis thaliana*. *In* AB Bennett, SD O'Niell, eds, Horticultural Biotechnology. Wiley-Liss, New York, pp305-314

Valvekes D, Lijsebettens MV, Van Montagu M (1988) *Arabidosis thaliana* root explant transformation and regeneration. PNAS 85, 5536-5572

Summary and Conclusions

The results from this body of work have helped establish the basic groundwork and provided the tools (namely the *cor* genes themselves) for future studies into how plants sense the cold, and the biochemical events that occur in order to bring about altered gene expression in response to low temperature. The fact that cold (or some physiological manifestation thereof) can have an effect on gene expression has been directly demonstrated by the cloning and study of the four cold-regulated genes. Further, data have been presented which provides strong evidence that there exist cis-acting promoter elements that can impart cold regulation to normally non-cold-regulated genes (such as GUS). Finally, the results from the studies on the kinetics of *cor78* induction and loss provide some evidence to suggest models concerning aspects of the signal transduction process(es) that may be operating in the induction of *cor78*. Briefly, it is possible (and perhaps most likely) that cold temperatures operate via a lengthy or slow signal transduction cascade. Alternatively, there could be a trans-acting factor that changes to an active conformation in a direct response to cold *per se*. However, for the data to be consistent with this last hypothesis, RNA polymerase II must be relatively inactive for nearly two hours after the plants reach inducing temperatures.

Much work however, is left to be done to further define the cold sensing mechanism(s) and the involvement of other genes and their proteins in the signal transduction pathway(s). There is now a some evidence that suggests that ABA levels increase during cold stress in *Arabidopsis* (Palva; Borg-Franck, personal communication). However, there is also some evidence to suggest that ABA may be operating independently of cold (and of drought) in the activation the *cor* genes (Nordin *et al.*, 1991; Gilmour and Thomashow 1991). Since G-box or G-box like sequences have been strongly correlated with ABA regulation, and all of the known *cor* genes from rice, rape and *Arabidopsis* contain G-box like sequences in their promoters (Mundy *et al.*, 1990; Singh *et al*, personal communication; Horvath *et al.*, unpublished results), it is likely that these sequences are having some effect on the ability of the *cor* genes to be expressed in response to ABA. In support of this, it has been shown that multimers of a particular G-box like sequence (hex3) has been shown to impart ABA and drought regulation to the CaMV 35S minimal promoter in tobacco (Lam and Chua, 1991). This demonstrates, at least in this case, that the G-box may be sufficient for ABA and drought regulation. However, in other deletion studies on light regulated genes, the G-box was shown to be necessary, but was not sufficient for appropriate light regulation of a reporter gene (Donald and Cashmore, 1990). Also, there are two known *Arabidopsis cor* genes (*cor15B* and *kin1*) that appear to have G-box like elements in their promoters, but which do not appear to be responsive to drought stress (Kurkela and Franck, 1991). Consequently, it is of considerable interest to determine if these G-box like sequences in the *cor* gene promoters are necessary and/or sufficient for cold regulation. Given these results, and the results concerning the expression of the *cor* genes in response to cold and drought in *abi1* mutant lines (Nordin *et al.*, 1991; Gilmour and Thomashow, 1991) it seems likely that the G-box like sequences will at least be necessary (if not sufficient) for the ABA regulation of the *cor* genes, but that they will not be sufficient (although they may prove necessary) for the regulation of these genes by cold *per se.*

Along with the G-box like sequences in the *cor* gene promoters, there is also another short GC rich sequence (CCGAC) that appears to be conserved. Not only is this sequence conserved among the *cor* genes, it is the only conserved sequence that is present in a 180bp fragment of the *cor15* promoter that appears to confer cold regulation to the CaMV 35S -90 promoter as determined by preliminary *in vitro* assays (McLarney, unpublished results). Therefore, it will be interesting to determine if multimers of this sequence can confer cold regulation to a minimal promoter, or if mutations or deletion of this sequence can prevent cold-regulated expression in the context of any of the known *cor* promoters.

In addition to looking at specific sequences and their presumed trans-acting factors, the constructs that have *cor* promoters driving GUS gene expression should provide excellent tools for studying other components of the signal transduction pathway that affect the *cor* genes. Plants carrying these constructs can be (and have been) mutagenized and screened for altered GUS gene expression, (either constitutive expression under control temperatures, or non-inducible expression in the cold) (Wilhelm, unpublished results). Such experiments should lead to the characterization and eventual isolation of the genes involved in the signal transduction pathway. Also, any presumed epistatic interactions of the resulting mutants should allow the elucidation of the steps and branch points in the signal transduction pathway(s).

From a more physiological aspect, the cloning of the *cor* genes make it possible to directly test the role (if any) that these genes have in protecting the plant from freezing damage. By freezing (without prior acclimation) plants that constitutively express these genes at warm temperatures, it should be possible to determine if any particular *cor* gene can, by itself, provide any protection against freezing stress. Also, utilizing antisense technology to reduce the function of any particular *cor* gene, or, if one were to obtain a mutant (see above) that was incapable of expressing any of the *cor* genes, it should be possible to determine if one or more of these genes are necessary for cold acclimation.

Finally, sequence data has shown that all of the *Arabidopsis* COR proteins are very hydrophilic in nature (Thomashow *et al.*, 1990). This finding is supported by the fact that they all remain soluble upon boiling (Lin *et al.*, 1990). This similarity suggests some possible commonality in the function of the COR proteins. However, much needs to be done in order to gain a better understanding of what the COR proteins are doing within the cells. Additional information gained concerning the intracellular location of these proteins, and the other types of molecules they associate or interact with (i.e. membranes, specific enzymes, etc.) could add immensely to the understanding of what these proteins do, and to their role (if any) in protecting the plants from freezing stress.

In the long term, the isolation of the *cor* genes and promoters may well prove quite valuable in the field of genetically engineered crop improvement. There are a number of agronomically important crop species (particularly in the family Solanacea) that are chilling tolerant but freezing sensitive and which have close relatives that are freezing tolerant (Chen *et al.*, 1983). Thus it is possible that these species have lost one or more of their own *cor* genes, or the ability to express them. Therefore, it may be possible to add one or more of the *Arabidopsis cor* genes (or genes necessary for their expression) to one of these crops and obtain individuals that are better able to handle freezing temperatures.

In plants in which the *cor* promoters are functional, (such as they are likely to be in *Brassica napus* particularly given the sequence similarities within the *cor* gene promoters of both *Brassica napus* and *Arabidopsis*), it should be possible to obtain lines that express any given gene in response to cold temperatures. For instance, it should be possible to place any gene, (such as genes that may be desirable to express during cold storage of produce), under the control of a *cor* promoter. Therefore, the *cor* promoters may prove to be useful tools for crop improvement.

At least one of the *cor* promoters has several advantages that make it amenable to various studies in basic plant biology. It has been established that the *cor78* promoter appears to rapidly shift from an induced state to a non-induced state when the plant is returned to warm temperatures (see Figure 3.10B). Also, it has been established that there are few changes in mRNA populations that occur in response to cold (Gilmour *et al.*, 1988). Therefore, the *cor* promoters should be useful in expression systems where expression under reasonably non-disruptive conditions, and/or rapid repression is desirable.

Finally, there is recent evidence for cross-talk between separate signal transduction pathways (as reviewed by Nishizuka, 1992), also, there may be several signal transduction pathways that play a role in the regulation of the *cor* genes (as discussed above). Consequently, these initial studies in the molecular biology of cold acclimation and cold-regulated gene expression, may provide a starting point for studies on the interaction and communication between the various signal transduction pathways involved in environmental sensing. The experiments and fields of study discussed above should add greatly to our understanding of plant gene regulation, biochemistry, and physiology.

Literature Cited

Brown, Ryan (1984) Isolation and characterization of a wound-induced trypsin inhibitor from alfalfa leaves. Biochemistry 23, 3418-3422

Chen HH, Li PH, Brenner ML (1983) Involvement of abscisic acid in potato cold acclimation. Plant Physiol. 71,362-365

Donald RGK, Cashmore (1990) Mutation of either G box or I box sequences profoundly affects expression from the *Arabidosis rbcS-1A* promoter. EMBO Journal 9, 1717-1726

Gilmour SJ, Hajela RK, Thomashow MF (1988) Cold acclimation in *Arabidopsis thaliana.* Plant Physiol. 87, 745-7750

Gilmour SJ, Thomashow MF (1991) Cold acclimation and cold-regulated gene expression in ABA mutants of *Arabidopsis thaliana.* Plant Mol. Biol. 17, 1233-1240

Kurkela S, Borg-Franck M (1992) Structure and expression of *kin2*, one of two cold- and ABA-induced genes of *Arabidopsis thaliana.* Plant Mol. Biol. 19, 689-692

Lam E, Chua N-H (1991) Tetramer of a 21-base pair synthetic element confers seed expression and transcriptional enhancement in response to water stress and abscisic acid. J. Biol. Chem. 266, 17131-17135

Lang V, Heino P, Palva ET (1989) Low temperature acclimation and treatment with exogenous abscisic acid induce common polypeptides in *Arabidopsis thaliana* (L.) Heynh. Theoretical and Applied Genetics 77, 729-734

Lin CT, Guo W, Everson E, Thomashow M (1990) Cold acclimation in *Arabidopsis* and wheat. Plant Physiol. 94, 1087-1083

Mundy J, Yamaguchi-Shinozaki K, Chua N-H (1990) Nuclear proteins bind conserved elements in abscisic acid-responsive promoter of a rice *rab* gene. PNAS 87, 1406-1410

Nishizuka Y, (1992) Signal transduction: crosstalk. TIBS 17, 367

Nordin K, Pekka H, Palva E (1991) Separate signal pathways regulate the expression of a low-temperature-induced gene in *Arabidopsis thaliana* (L.) Heynh. Plant Mol. Biol. 16 1061-1071

Thomashow M, Gilmour S, Hajela R, Horvath D, Lin C, Guo W (1990) Studies on cold acclimation in *Arabidopsis thaliana.* IN, AB Bennett, SD O'Neill, eds, Horticultural Biotechnology. Wiley-Liss, New York, pp305-314

APPENDIX

Correlation of freezing tolerance to *accumulation of cor* gene transcripts

For some time, it has been known that when the plants undergo cold acclimation, *cor* genes expression is induced. Also, other work had demonstrated that environmental conditions other than cold could bring about the induction of the *cor* genes (Chen *et al.*, 1983). For instance, it was shown that exogenous application of ABA to plate grown *Arabidopsis* plants could both induce the *cor* genes and increase the cold hardiness of the treated plants (*Lang et al.*, (1989). It was of interest then to determine if any other environmental conditions that bring about the induction of the *cor* genes would also be able to increase the freezing tolerance in plants. A series of experiments were undertaken to correlate the levels of freezing tolerance to the expression of the *cor* genes. To do this, the level of freezing tolerance in plants was determined after they were subjected to treatments known to induce the *cor* genes (5°C, 10°C, drought stress, and salt stress), or to decrease the expression of already induced *cor* genes (deacclimation).

In initial studies on the freezing tolerance of *Arabidopsis* using electrolyte leakage as an indicator of tissue damage caused by freezing, it was determined that unacclimated *Arabidopsis* exhibited a loss of 50% (Lt_{50} for lethal temperature 50) of their electrolytes at around -3°C, but that *Arabidopsis* which had been acclimated at 5°C for three days exhibited an Lt_{50} at about -5°C to -7°C (Gilmour *et al.*, 1989). Similar results were obtained in these experiments (Figures A.1-4). The data obtained from plants that were exposed to 10°C, or drought stress or salt stress, did not appear to significantly change the Lt_{50} from that of the controls (Figures A.1-3). This data however, does not suggest that these plants are better able to survive a freezing stress. It should be noted, that in both the drought stressed and the salt stressed plants, the initial electrolyte leakage at -1°C was abnormally high (around 40%) as compared to the cold-treated and control plants (which averaged around 20%), suggesting that these plants were significantly damaged by the stresses (Figures A.2 and A.3). It should also be noted that both drought and salt stressed plants exhibited 100% electrolyte leakage at temperatures significantly lower than the controls (-8°C as compared to -6°C) and only one degree lower than that of plants which were fully acclimated (3 days at 5°C) (figures A.2 and A.3). This last observation may indicate that drought and salt stress may indeed increase the freezing tolerance of *Arabidopsis*. Therefore, taken together with the results of the ABA treatment from Palva (1989), these results are consistent with the idea that the expression of the *cor* genes correlates with an increase in the freezing tolerance.

In contrast to these results, treatment of the plants at 10°C for two days did not appear to have a significant effect on the freezing tolerance of the plants (Figure A.1) Since it is likely that these plants were expressing the *cor* genes, it appears that simple accumulation of the cor mRNA does not always correlate with increased freezing tolerance. In fact, it is common practice to acclimate plants for greater than three days in order to get very reproducible differences in electrolyte leakage between acclimated and nonacclimated plants (Artus and Gilmour, unpublished observations). It should also be noted, however, that although the *cor* genes were likely to be induced, the actual accumulation of the COR proteins during any of these experiments was not determined.

Results from earlier experiments indicated that the level of *cor* gene message in deacclimating plants begins to drop within 1 hours and return to control levels within

Figure A.1 Freezing tolerance of salt stressed plants. Plants were cold treated at 5^{o}C for 3 days (◆), left untreated (■), or watered with 200 mls of a 0.4 M NaCl solution and allowed to sit overnight (▲). Leaves were then removed at random, and the percent of electrolyte leakage (relative to the same leaf samples after freezing to -80^{o}C) was measured after freezing to various temperatures according to the methods of Gilmour *et al.*, 1988).

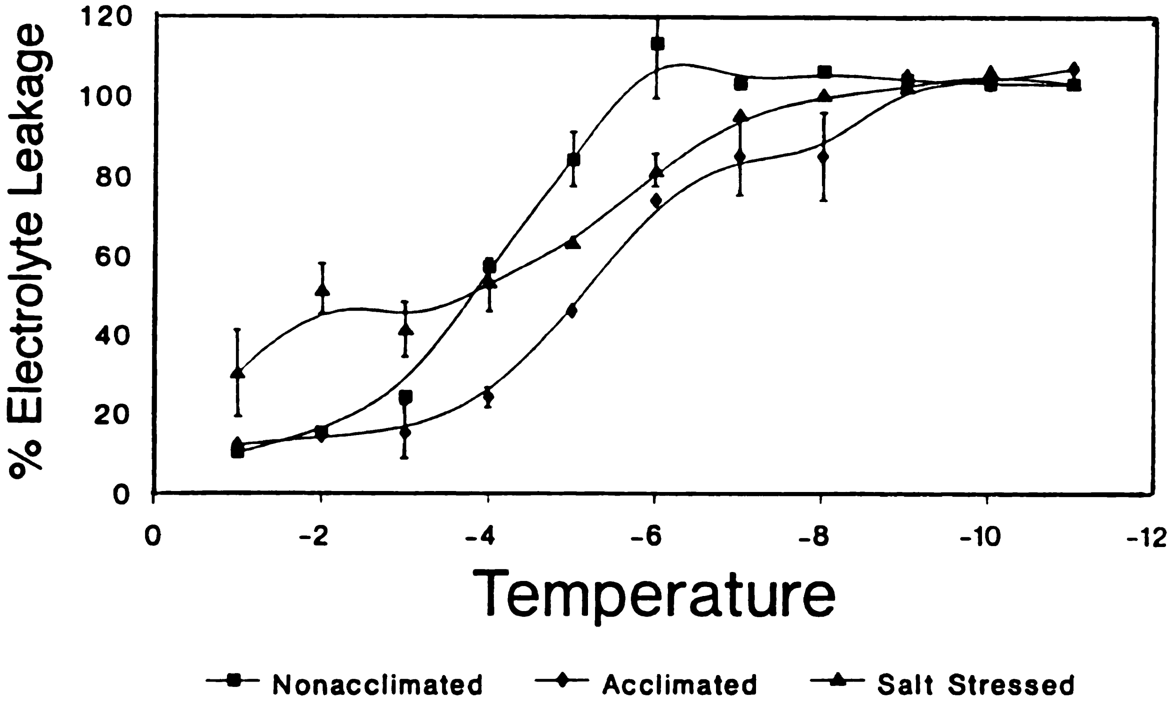

% Electrolyte Leakage
120
100
80
60
40
20
0
0
-2
-4
-6
-8
-10
-12
Temperature
Nonacclimated
Acclimated
Salt Stressed

Figure A.1

Figure A.2 Freezing tolerance of drought stressed plants. Plants were cold treated at $5^{o}C$ for 3 days (◆), left untreated (■), or were left unwatered until they reached a relative water content below 68% (▲). Leaves were then removed at random, and the percent of electrolyte leakage (relative to the same leaf samples after freezing to $-80^{o}C$) was measured after freezing to various temperatures according to the methods of Gilmour *et al.*, 1988).

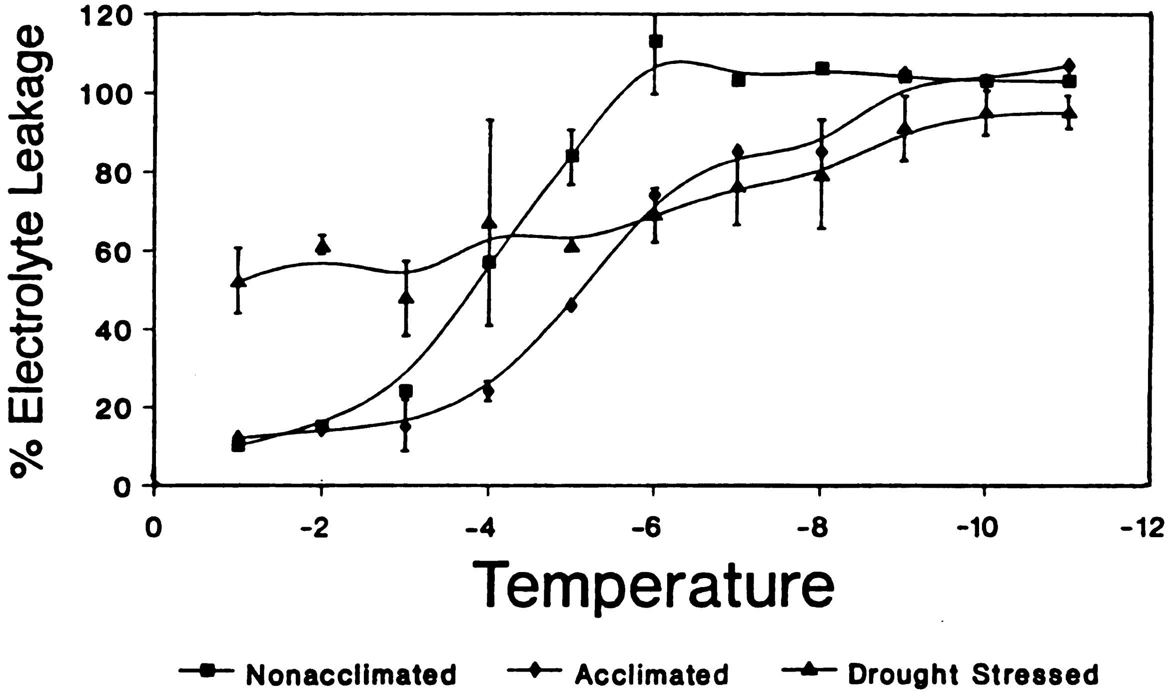

% Electrolyte Leakage
120
100
80
60
40
20
0
0
-2
-4
-6
-8
-10
-12
Temperature
Nonacclimated
Acclimated
Drought Stressed

Figure A.2

Figure A.3 Freezing tolerance of plants cold acclimated at 10^{O}C. Plants were cold treated at 5^{O}C for 3 days (♦), left untreated (■), or were placed at 10^{O}C for 2 days (▲). Leaves were then removed at random, and the percent of electrolyte leakage (relative to the same leaf samples after freezing to -80^{O}C) was measured after freezing to various temperatures according to the methods of Gilmour *et al.*, 1988).

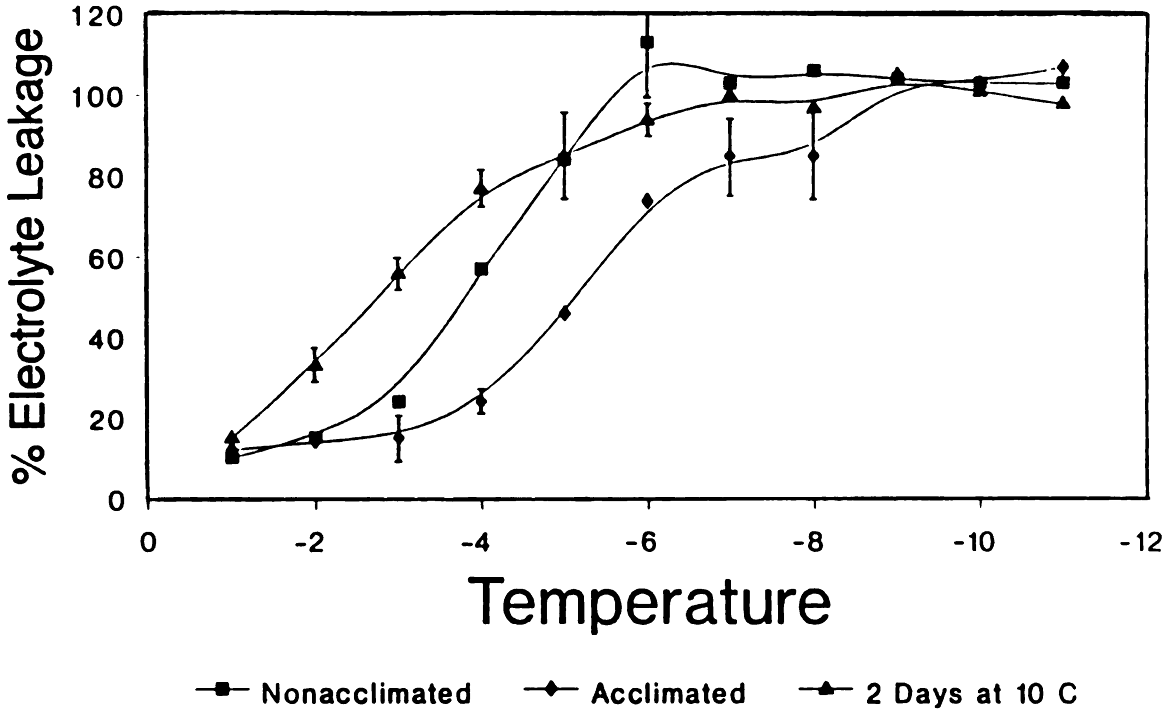

% Electrolyte Leakage
120
100
80
60
40
20
0
0
-2
-4
-6
-8
-10
-12
Temperature
Nonacclimated
Acclimated
2 Days at 10 C

Figure A.3

Figure A.4 Freezing tolerance of plants after cold acclimation for various lengths of time, and for various lengths of time after deacclimation. Plants were cold treated at 5°C for from 0 to 20 days, (solid line), or moved from the cold after 14 days and left to deacclimate (dotted line). Leaves were then removed at random, and the percent of electrolyte leakage (relative to the same leaf samples after freezing to -80°C) was measured after freezing to various temperatures according to the methods of Gilmour *et al.*, 1988). The point at which plants from each treatment lost 50% of their electrolytes (LT_{50}) was then plotted over time.

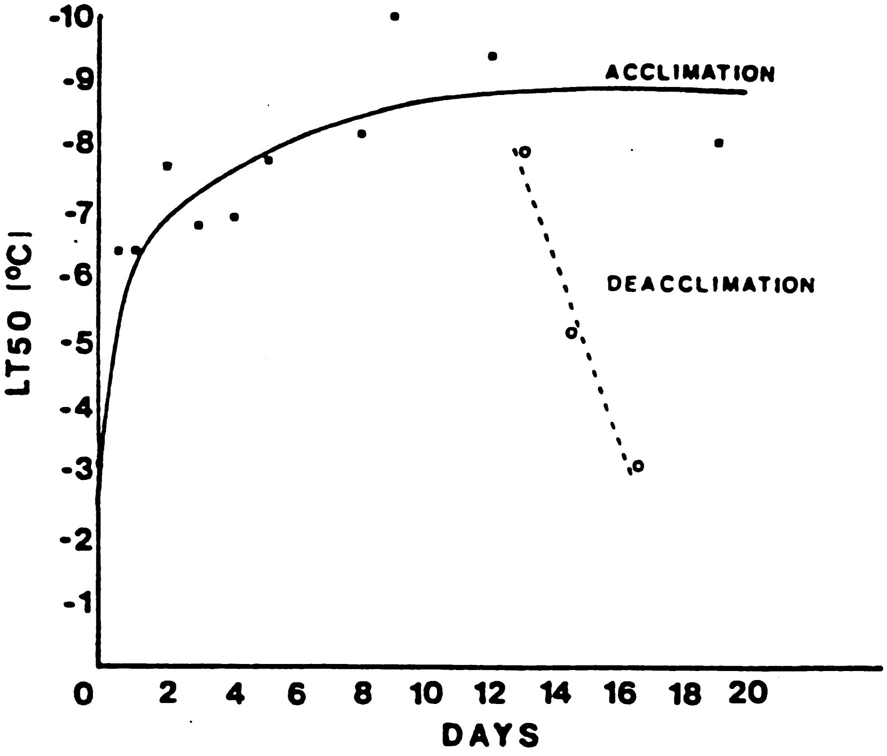

-10
-9
-8
-7
-6
-5
-4
-3
-2
-1
LT50 (°C)
0
2
4
6
8
10
12
14
16
18
20
DAYS
ACCLIMATION
DEACCLIMATION

Figure A.4

8 hours after plants are moved out of the cold (Hajela *et al.*, 1990). Therefore, it was of interest to determine if the loss of *cor* gene activity correlated with the loss of freezing tolerance. The results in this experiment demonstrated that deacclimating plants still maintain a significant level of freezing tolerance up to two days after they were moved back normal growing temperatures (Figure A.4). This may suggest that the expression of the *cor* genes is not absolutely necessary for the maintenance of freezing tolerance. However, the stability of the *cor* gene products is not known. Therefore, it is possible that the COR proteins remain at relatively high levels for some time after the plants are returned to normal growing temperatures. Further studies should be done to determine the half-life of the COR proteins, in order to clarify this conflict.

Kinetics of expression of the *cor78*/GUS gene construct

Once it was determined that the region of *cor78* between -808 and +5 could impart cold regulation to a GUS reporter gene (see Figure 3.5B and 3.5C), it was of interest to determine if these sequences could also impart the same kinetics of induction and loss of the GUS reporter gene as was seen with the endogenous *cor78*. Consequently, an *Arabidopsis* line carrying the *78Pgus* construct was grown on plates for 18 days and then placed at 5^{o}C for either 3 hours or overnight. Plates were placed back at room temperature and samples were taken at various times for RNA extractions and northern analysis (Figure A.5-8).

Preliminary results indicated that indeed, similar kinetics of induction and loss of the GUS message could be obtained by placing the GUS gene under the control of the *cor78* promoter (Figure A.5-8. However, a few minor differences in the

Figure A.5 Northern analysis of the kinetics of *cor78* expression. Plate grown *Arabidopsis* plants containing the *cor78/gus* construct were placed at $3^{o}C$ for 3 hours and then moved back to room temperature. Plants were harvested every fifteen minutes from 0 minutes to 60 minutes and then every half hour for the last two time points. RNA was then harvested from each time point, and run out on a gel for northern analysis. Gels were blotted and probed with ^{32}P-labeled *cor78* clone. Hybridization was quantified on a B-scope 3000. The counts were normalized as a percentage of the maximum counts in the hottest lane. The log of the percent accumulation was then plotted over time.

te grown
for 3 hours
fifteen
st two time
gel for
clone.
alized as a
rcent

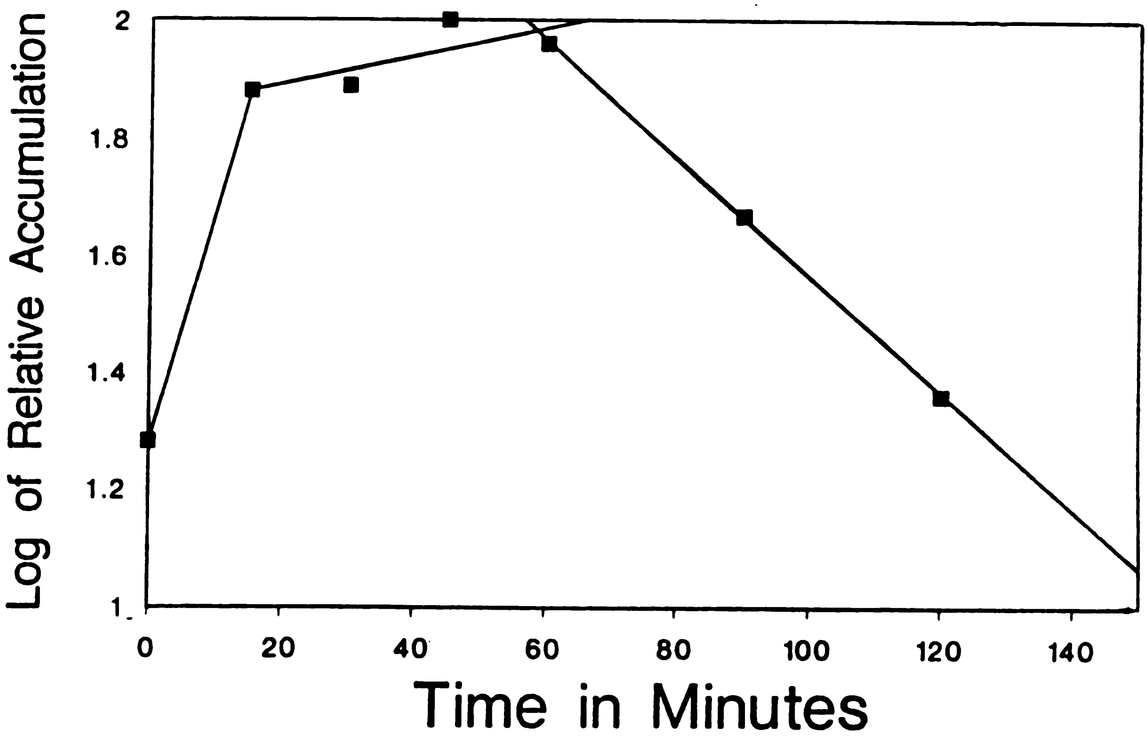
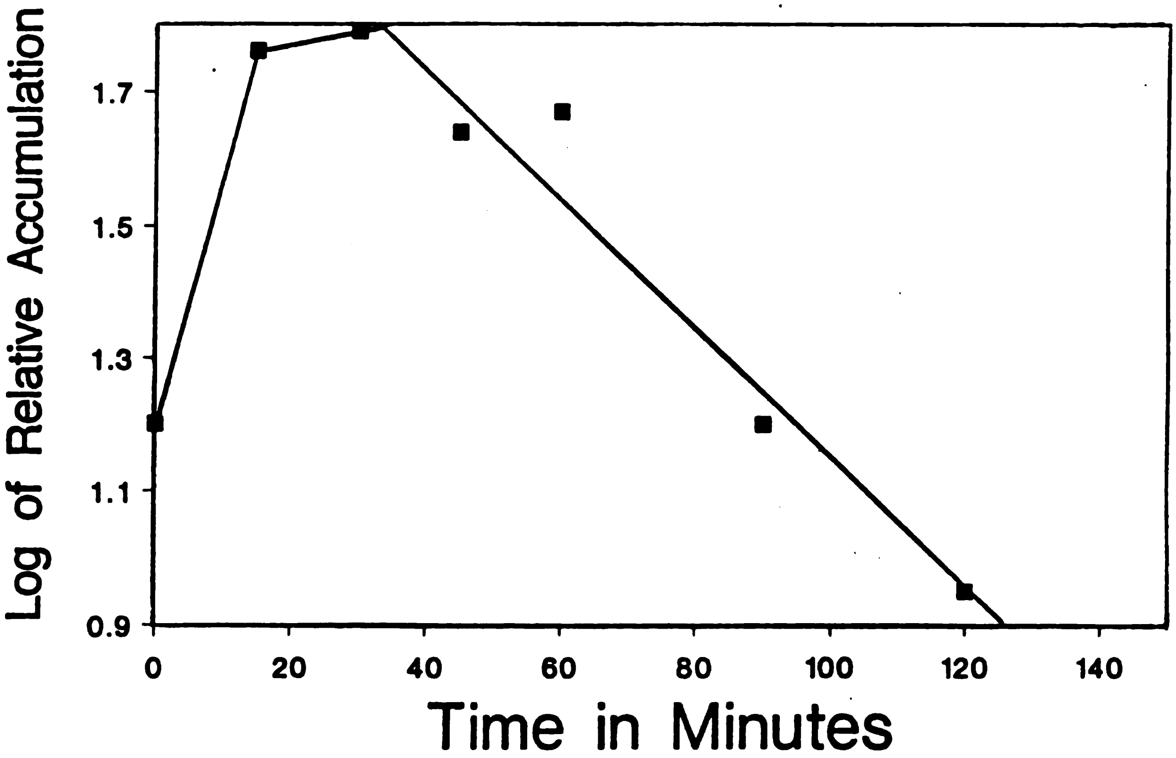


Figure A.5

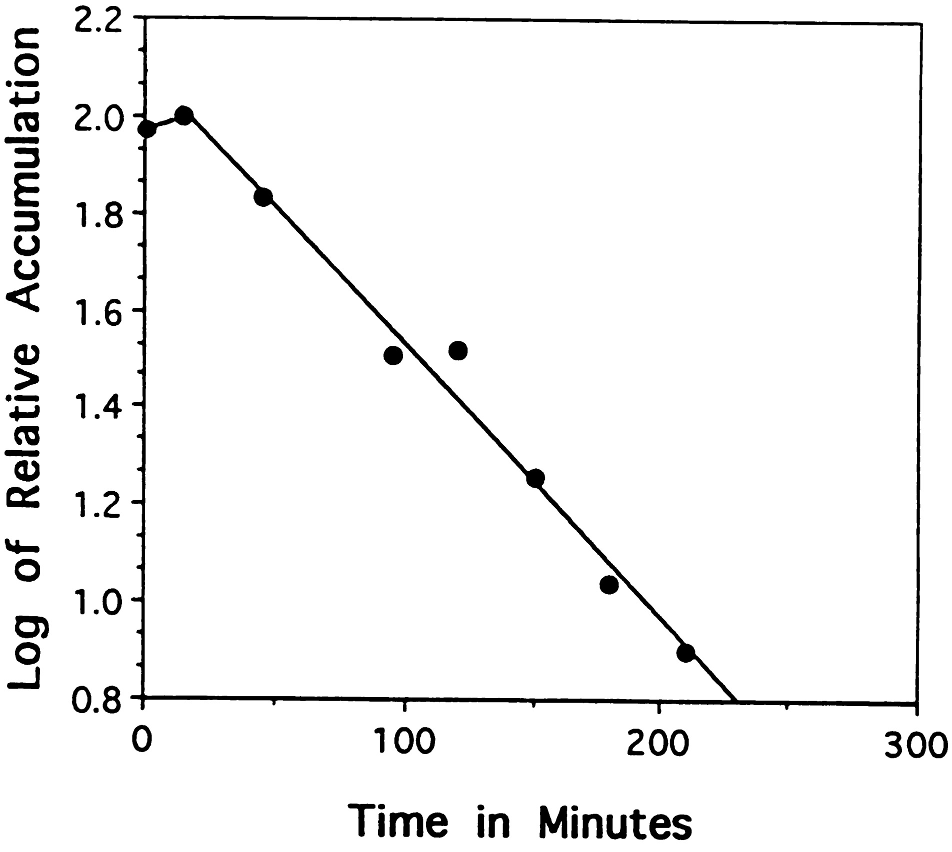
Figure A.6 Northern analysis of the kinetics of GUS expression. Plate grown *Arabidopsis* plants containing the *cor78/gus* construct were placed at 3^{o}C for 3 hours and then moved back to room temperature. Plants were harvested every fifteen minutes from 0 minutes to 60 minutes and then every half hour for the last two time points. RNA was then harvested from each time point, and run out on a gel for northern analysis. Gels were blotted and probed with ^{32}P-labeled GUS gene isolated from pBI101. Hybridization was quantified on a B-scope 3000. The counts were normalized as a percentage of the maximum counts in the hottest lane. The log of the percent accumulation was then plotted over time.


Log of Relative Accumulation
1.7
1.5
1.3
1.1
0.9
0
20
40
60
80
100
120
140
Time in Minutes

Figures A.6

Figure A.7 Northern analysis of the kinetics of *cor78* expression. Plate grown *Arabidopsis* plants were placed at 3^{o}C overnight and then moved back to room temperature. Plants were harvested every at various times. RNA was then extracted from each time point, and run out on a gel for northern analysis. Gels were blotted and probed with the *cor78* clone. Hybridization was quantified on a B-scope 3000. The counts were normalized as a percentage of the maximum counts in the hottest lane. The log of the percent accumulation was then plotted over time.

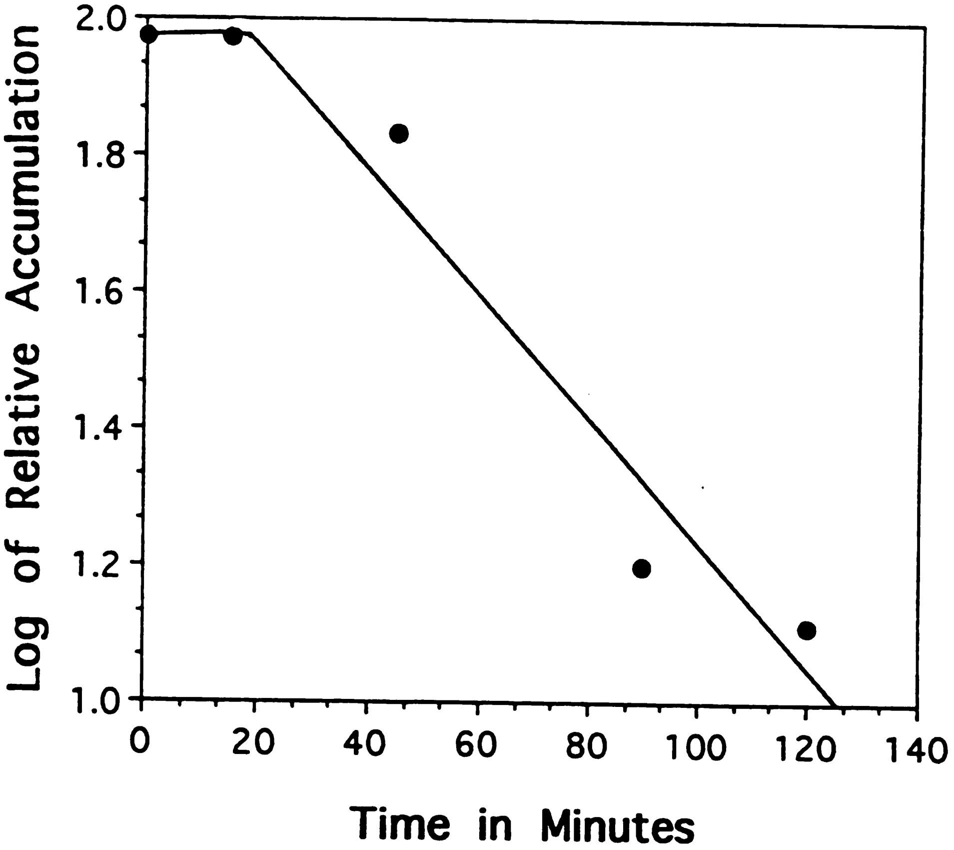
:ate grown
to room
:hen extracted
were blotted an
be 3000. The
hottest lane.



Figures A.7

Figure A.8 Northern analysis of the kinetics of GUS expression. Plate grown *Arabidopsis* plants were placed at 3^{o}C overnight and then moved back to room temperature. Plants were harvested every at various times. RNA was then extracted from each time point, and run out on a gel for northern analysis. Gels were blotted and probed with the GUS gene isolated from pBI101. Hybridization was quantified on a B-scope 3000. The counts were normalized as a percentage of the maximum counts in the hottest lane. The log of the percent accumulation was then plotted over time.

ate grown
o room
then extracted
were blotted a
uantified on a B
um counts i
over time.



Figures A.8

accumulation and loss curves were detected. In this one experiment, it appeared that the GUS message only continued to accumulate for 30 minutes after the plants were moved back to normal growing temperatures following a three hour cold treatment (Figure A.6). The endogenous *cor78* gene, however, continued to accumulate for 45 to 60 minutes in the same plant samples (Figure A.5). Further experiments would need to be done in order to determine if this slight difference in induction was repeatable. Another difference that was observed was that the loss of the GUS message appeared to be significantly slower (Figure A.8) than that from the endogenous *cor78* (Figure A.7). Given that the half-life of the *cor78* message is unusually short, it is not surprising that the GUS gene may be more stable and therefore have somewhat different kinetics of degradation.

Literature Cited

Chen HH, Li PH, Brenner ML (1983) Involvement of abscisic acid in potato cold acclimation. Plant Physiol. 71,362-365

Gilmour SJ, Hajela RK, Thomashow MF (1988) Cold acclimation in *Arabidopsis thaliana*. Plant Physiol. 87, 745-7750

Hajela R, Horvath DP, Gilmour SJ, Thomashow MF (1990) Molecular cloning and expression of *cor* (cold-regulated) genes in *Arabidopsis thaliana*. Plant Physiol. 93, 1246-1252

Lang V, Heino P, Palva ET (1989) Low temperature acclimation and treatment with exogenous abscisic acid induce common polypeptides in *Arabidopsis thaliana* (L.) Heynh. Theoretical and Applied Genetics 77, 729-734

Lin CT, Thomashow (1992) DNA sequence analysis of a complimentary DNA for cold-regulated *Arabidopsis* gene *cor15* and characterization of the *COR15* polypeptide. Plant Physiol. 99,