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STUDIES ON LOW TEMPERATURE INDUCED GENE REGULATION AND FREEZING STRESS TOLERANCE IN *ARABIDOPSIS*

By

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ABSTRACT

STUDIES ON LOW TEMPERATURE INDUCED GENE REGULATION AND FREEZING STRESS TOLERANCE IN *ARABIDOPSIS*

By

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Low temperature is a significant environmental stress factor limiting growth and productivity of agronomically important plants in many parts of the world. Plant responses to low temperature are complex, involving coordinated regulation of multiple biochemical pathways leading to expression of a number of genes encoding proteins that contribute to cold acclimation. Genes whose expression is increased during low temperature stress include those associated with freezing stress as well as the cellular protective enzymes, proteins and other compounds involved in osmotic adaptation to and tolerance of the cellular dehydration caused by freezing stress. Other up regulated genes include the several signaling proteins such as protein kinases and transcription factors that play roles in adapting plants to tolerate freezing temperature. Some of the regulatory DNA sequences that confer responsiveness to low temperature and associated stresses have been identified and transcription factors that interact with such *cis*-elements have been partially characterized. Work to identify additional sequences and factors involved in the sensation of low temperature and the transduction of the earliest gene response signals is continuing through the use of reverse genetics and mutagenesis studies. In particular, the following

describes efforts to characterize some of the functions of the *CBF* genes as transcriptional activators in the low temperature signal transduction pathway and also to identify sequences responsible for regulation of their own activation. Through the use of promoter deletion analyses, a 155bp fragment of the *CBF2* promoter was found to be responsible for low temperature inducible expression of the gene as well as responsiveness to mechanical agitation, ABA and the translation inhibitor cycloheximide. Some transcript accumulation at warm temperatures in the presence of cycloheximide also led to the idea that an inhibitor of *CBF* as well as an inducer of *CBF* expression (ICE) may be working to precisely regulate *CBF* expression.

Work to identify additional genes associated with the low temperature signal transduction pathway upstream of the *COR* genes using a *COR* gene promoter and marker gene fusion system and chemical mutagenesis was performed. Markers included the *BAR* gene, *codA*, and the *R* gene. Several mutants affected in their expression of both the *R* gene marker and one of the *COR* genes were isolated but the change in expression was likely due to gene silencing and not interruption of the cold signaling pathway. Plants exhibiting loss of the endogenous *COR78* expression were examined with an ion leakage assay to assess the significance of the loss of this one *COR* gene on freezing tolerance. No significant change was seen in these plants, however, a wealth of information was obtained concerning the use of different reporter genes and possible methods to improve the search for genes.

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TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1

PLANT COLD ACCLIMATION AND FREEZING STRESS TOLERANCE

Introduction

Anchored in the environment in which they germinate, plants are often subjected to adverse growth conditions including extremes in temperature, drought and salinity. To survive, plants have developed a complex signaling system that allows them to sense and respond to an ever-changing environment. Tolerance of freezing is considered a primary limiting factor for growth, productivity and distribution for many important crop plants and wild species in the northern latitudes throughout North America, Europe, Asia and the high altitudes of South America. Considerable time and energy has been directed toward efforts to enhance freezing tolerance in agriculturally important plants. However, limited advancement has been achieved in the progress toward increasing freezing stress tolerance in most plants due in part to the complexity of the problem that is being addressed. Multiple stress factors, both biotic and abiotic are often occurring at the same time and affect plants that reside in habitats that experience freezing temperatures. Understanding these influences individually is difficult enough but the problem is exacerbated by unknown roles of the potentially hundreds of genes involved in the acquisition of freezing tolerance and the need to battle the multiple stresses associated with a low temperature environment.

Freezing temperature is one part of a set of stresses that must be tolerated in order to survive in an environment that frequently produces sub-optimal growth temperatures. For many plants growing in temperate zones, environmental factors such as wind and ice formation can increase the tendency for dehydration. Water logging and ice encasement cause anaerobic stresses. High light combined with low temperature can lead to photoinhibition. In addition plants that over winter may be subject to several diseases (Gaudet, 1994; Tronsmo *et al.*, 1993; Wise 1995).

Tolerance of these low temperature stresses can vary considerably between species of plants. If one considers just the effect of cold temperatures, plants can be classified into different groups based on their ability to cope. Most tropical plants and many important crop plants such as rice and corn show injury or/and loss of viability at temperatures between 0°C and 15°C and are considered chilling sensitive (Anderson *et al.*, 1994; Taylor *et al.*, 1974). Plants such as cultivated potato (*Solanum tuberosum*) can tolerate low nonfreezing temperatures but are injured or killed when ice begins to form in their tissues (Sukumaran and Weiser, 1972). Other plants can survive ice formation in their tissues but are killed by high sub-zero temperatures. For example, many types of citrus plants are injured or killed at temperatures of -3°C to -6°C (Yelenosky and Guy, 1989). When acclimated, many of the cereals and temperate herbaceous species of plants can survive freezing to temperatures ranging from -10°C to -30°C (Fowler and Gusta, 1979; Scorza *et al.*, 1983). Some of the most

hardy woody perennials can survive -30°C to -50°C in nature or even immersion in liquid nitrogen (-196°C) when fully acclimated (Sakai, 1960).

In plants with the same genetic potential to acclimate to freezing temperature, variations can be seen in the maximum degree to which a single individual or type of plant can acclimate depending on the immediate surroundings of the plant or other stresses affecting its growth. For example, depending on growth conditions from year to year, some types of winter wheat may have a maximal tolerance that varies from -16°C to -24°C (Fowler *et al.*, 1983). Lengthening the time over which the plant acclimates can often increase the degree of tolerance. Although time is only one of many factors contributing to tolerance, the longer the acclimation period the more likely a plant will be able to reach its maximum tolerance level.

Because the ability to gain freezing tolerance requires time, even the most freezing tolerant species may be sensitive to low temperatures in a nonacclimated state such as in the spring after growth has resumed or until growth stops in late summer or fall. At the end of a growing season however, plants respond to cues such as decreasing temperature and changes in day length in such a way as to increase their freezing tolerance, which allows them to survive the prolonged periods of low temperatures. This adaptive change leading to an increase in tolerance to freezing has been termed cold acclimation. Tolerance to freezing that is gained by cold acclimation is transient though and is rapidly lost during environmental conditions in which temperatures rise above freezing. Therefore cold acclimation can be considered a dynamic physiological

process that increases the level of a plant's tolerance to low temperature to different extents depending on the level of conditioning prior to the freezing stress. The level of acclimation is determined by factors such as the rate of cooling, the minimum temperature reached and duration at that temperature, the rate of thawing and interaction with other stresses in the environment. The genetic potential and physiological strategies used to survive these stresses vary greatly between plants. For some plants, preventing freezing by allowing water within the tissues to supercool without forming ice crystals increases the chance of survival. Others form ice but prevent its spread with barriers or prevent movement of water from within cells out to the ice. Mechanisms to survive dehydration are important because once extracellular ice is formed, the crystal grows by drawing water from the cells. Because the water potential of ice is lower than that of liquid water within the cell, water moves down a chemical potential gradient from inside the cell to extracellular spaces which leads to the cellular dehydration stress. Therefore, factors that influence a plant's ability to survive freezing temperature include whether supercooling or ice formation occurs, the location of ice formed, whether mechanisms exist to prevent the spread of ice or the movement of water to ice and the ability to tolerate dehydration stresses.

Freezing, dehydration and damage

When the air or soil temperatures drop below the freezing point of water, ice crystals can begin to form in plant tissues. Ice normally forms in vessels of the xylem in leaves and stems and intercellular spaces first due to relative

negative osmotic potential of the sap. The temperature required for formation of ice, even here, may be several degrees below 0°C because the water molecules need a nucleation site to first start forming the ice crystals. Location of nucleation sites in plant tissues can be controlled by the composition and structure of cell walls. Once ice has formed, subsequent nucleation occurs on the surface of the ice crystal itself. Ice crystals then spread throughout the vessels and extracellular spaces. As the ice crystal grows and accumulates water, it excludes the solutes and these accumulate in the remaining liquid. Ice formation continues until the chemical potential of the unfrozen water is in equilibrium with the ice (Mazur, 1970). Because solutes are excluded from ice, the extracellular unfrozen solution becomes more concentrated. This creates a gradient in the water potential between intracellular and extracellular solutions, which causes water to move out of the cytoplasm until water potentials equilibrate again. This results in cytoplasmic dehydration.

In a nonacclimated cell after ice nucleation has occurred, large amounts of water cross the plasma membrane. As ice forms, large crystals cause tissues to swell and the cytoplasm experiences dehydration, which may be extreme. Damage resulting from freezing most often will result in death for the cell. Acclimation reduces damage through several adaptive changes that take place in the cell such as increasing the solute concentration within the cytoplasm to decrease water movement out of the cell, changing the composition of the plasma membrane to tolerate freeze thaw processes and avoidance of freezing in the first place by supercooling. Supercooling is achieved in plants by

minimizing nucleation sites. It avoids the damage caused by cytoplasmic dehydration, however, the protection cannot exceed the critical threshold for ice nucleation, which is about -38°C. The degree of success minimizing nucleation sites determines the temperature to which water in a particular plant or tissue can supercool.

While many types of damage to cells can affect viability, the immediate target of freezing induced damage appears to be the membrane systems in plant cells (Pearce and Willison, 1985; Steponkus, 1984). Many forms of membrane damage have been recognized as a consequence of freezing induced cellular dehydration and therefore a key function of the cold acclimation process is to stabilize membranes. Damage can result from expansion-induced lysis or local changes in phase from lamellar to non-lamellar (Pearce, 1985; Pearce and Willison, 1985; Pearce, 1988; Steponkus and Webb, 1992; Steponkus *et al.*, 1993; Uemura *et al.*, 1997). In the case of expansion-induced lysis, as water diffuses out of the cell, extracellular ice forms. The plasma membrane loses tension and through a process of endocytotic vesiculation loses parts of the membrane. When the cell thaws, the influx of additional water causes expansion and eventual lysis of the cell (Steponkus, 1984).

Non-lamellar, or hexagonal II phase transitions result from the destabilization of membrane protein lipid interactions (Williams, 1990). The appearance of fracture-jump lesions, identified through the use of a freeze fracture electron microscopy technique, is an indication of changes in lipid interactions in the membrane bilayer (Webb and Steponkus, 1993). Membrane

stabilization can be aided by changes in lipid composition (Steponkus *et al.*, 1993; Uemura *et al.*, 1997). Other soluble molecules including carbohydrates that accumulate during growth at low temperature can also stabilize membranes (Strauss and Hauser, 1986; Crowe *et al.*, 1992; Anchordoguy *et al.*, 1987) and thus help cells survive.

Other potential effects resulting from freezing include inhibition of catalytic activity of multimeric enzymes due to dissociation of its subunits and loss of conformational stability at temperatures below the freezing point of water leading to protein denaturation (Li *et al.*, 1999). Certain molecular chaperones may play roles in maintaining conformation and assembly of proteins during freezing stress and thus allow tolerance of the stress. Members of the family of HSP70 protein molecular chaperones are influenced by low temperature. Increases in expression could be due to a decrease in temperature or due to the loss of cellular homeostasis caused by processes affected by low temperature, such as impairment of protein biogenesis or cold denaturation of cold labile proteins. Increases in expression are most pronounced at chilling temperature but several of the stress 70 and other molecular chaperones are upregulated several hours after a freeze/thaw stress (Li *et al.*, 1999; Anderson *et al.*, 1994; Parswell and Lindquist, 1993).

Oxidative stress tolerance is also an important component of freezing tolerance. Reactive oxygen species can be deleterious to membranes and photosystems (Tanino and McKersie, 1985; Wise, 1995). Enhancement of antioxidative mechanisms may minimize damage and are normally expressed at

higher levels in low temperature stressed plants. McKersie *et al.* (1993) have shown that transgenic alfalfa that over expressed Mn-superoxide dismutase (SOD) cDNA had enhanced freezing tolerance and higher growth rate after a sub-lethal freezing stress. Less benefit was seen after more severe freezing temperature and higher levels of active oxygen indicating the SOD system may have been overwhelmed. The levels of other potential oxidative stress protective enzymes including ascorbate peroxidase, glutathione reductase and catalase (Bridger *et al.*, 1994; Hull *et al.*, 1997; Prasad, 1997; Prasad *et al.*, 1994; Jahnke *et al.*, 1991; Walker and McKersie, 1993) increase after exposure of cells to low temperature. Working in combination these may help reduce the damage caused to photosynthetic systems by reactive oxygen molecules (Krivosheeva *et al.*, 1996).

As previously stated, cold acclimation in plants is an adaptive response to low non-freezing temperatures that leads to an increase in tolerance to below freezing temperatures. In addition to low temperature, dehydration stress can lead to an increase in freezing tolerance (Guy *et al.*, 1992a). This is not surprising since during periods of freezing, extracellular ice formation causes cellular dehydration. Analysis of the major water stress induced genes and the low temperature induced genes reveals similarities in the families of genes that are induced. One of the major families is the late embryogenesis abundant or LEA. Many of the Arabidopsis *COR* genes fall into this family and will be discussed later. Dehydration stress also results in the endogenous synthesis and accumulation of abscisic acid (ABA) (Write and Hiron, 1969) and may trigger

the acclimation response. ABA levels are known to increase in a variety of plants during cold acclimation. This led to the hypothesis of Chen and Gusta (1983) that cold acclimation is activated through an ABA mediated pathway. This idea was further strengthened with the observation that exogenous applications of ABA to plants kept at warm temperatures enhanced their freezing tolerance (Chen *et al.*, 1983; Ishikawa *et al.*, 1990; Orr *et al.*, 1986). And with the commonality of molecular responses and genes associated with drought and cold acclimation, ABA appeared to be a common agent that mediated responses to both types of stresses (Close *et al.*, 1989; Gilmour *et al.*, 1992; Horvath *et al.*, 1993; Nordin *et al.*, 1993; Yamaguchi-Shinozaki and Shinozaki, 1993a). In addition, *Arabidopsis* plants carrying mutations in ABA synthesis (*aba1*) or in the plant's ability to respond to ABA (*abi1*) are less freezing tolerant than wild type (Gilmour and Thomashow, 1991; Heino *et al.*, 1990; Mäntylä *et al.*, 1995). However, ABA does not seem to be required for activation of several cold responsive genes since their expression is still induced by low temperature in both the *aba1* and *abi1* mutants (Gilmour and Thomashow, 1991; Nordin *et al.*, 1991). The discovery of separate *cis*-acting DNA regulatory elements in the promoters of cold responsive genes also supports the idea of independent cold induced and ABA regulated pathways (Baker *et al.*, 1994; Yamaguchi-Shinozaki and Shinozaki, 1994). Ishitani *et al.* (1997), isolated mutants that increase or decrease expression of cold regulated genes in response to both cold and ABA. These results suggest that the cold and ABA regulatory pathways are not completely independent pathways but are rather part of a network that shares

some components. Identification of more factors in the signal transduction pathways should help clarify the relationship between cold and ABA.

Regardless of the mechanism by which the low temperature signal is transmitted, certain facts about the cold acclimation process are known. For instance, during the acclimation process, a plant's metabolism changes considerably. Rates of respiration, photosynthesis and synthesis of proteins and other compounds are all up-regulated (Guy, 1990). Solutes accumulate during low temperature growth that can change osmotic potential, which influences the freezing or ice nucleation point in cells. The major changes in osmotic potential are due to changes in sugars (Levitt, 1980; Sakai and Larcher, 1987). Some studies on plants that demonstrate this include the observation that an increase in soluble sugar content of cabbage leaves, led to acquisition of freezing tolerance. The slow increase of sucrose, glucose and fructose correlates with the degree of freezing tolerance. The loss of freezing tolerance after one day of deacclimation is associated with large reductions in sugar content (Sasaki *et al.*, 1996). In alfalfa, the accumulation of sucrose, stachyose and raffinose correlates with development of freezing tolerance. In addition, differences in freezing tolerance between a cold tolerant and a cold sensitive cultivar are closely associated with accumulation of raffinose and stachyose (Castonguay *et al.*, 1995). In winter cereals, fructans are storage carbohydrates that act to regulate sucrose levels and provide osmoregulation. In response to freezing they are hydrolyzed into soluble sugars, which provided some protection against freezing (Olien and Clark, 1995; Livingston and Henson, 1998).

Membrane lipid composition also plays an important role in cold acclimation. Early work showed that as plants acclimated to low temperature, lipids became more unsaturated (Lynch and Steponkus, 1987; Steponkus, 1984). More recently, the composition of lipids that make up the membranes has been shown to change as a result of acclimation to cold. In *Arabidopsis thaliana*, the proportion of phospholipids increase in percent over total lipids while the proportions of cerebrosides and free sterols decrease (Uemura *et al.*, 1995). In cold acclimated winter rye and oats, the proportions of phospholipids increase significantly as a result of increases in proportions of phosphotidylcholine and phosphotidylethanolamine. The relative proportion of diunsaturated species also increases and there is a decrease in the proportion of cerebrosides in the plasma membrane (Lynch and Steponkus, 1987; Uemura and Steponkus, 1994). These changes all contribute to the increase in freezing tolerance that occurs during acclimation (Steponkus *et al.*, 1988).

Cold up-regulated genes

In 1970, Weiser suggested that cold acclimation required transcriptional activation of a set of genes that are not normally expressed under non-cold acclimating conditions. Guy *et al.* (1985) were the first to demonstrate the accuracy of this supposition when they showed that exposure of spinach leaves to low temperature (5°C) led to induction of newly translatable mRNAs. A number of plant genes have since been isolated from both monocotyledonous and dicotyledonous species that are up-regulated in their mRNA levels by low temperature treatments. Many of these genes encode proteins with known

enzymatic activities that may contribute to freezing tolerance. They include some relating to primary metabolism or stress metabolism such as the housekeeping genes β-tubulin (Chu *et al.*, 1993) and *EF-1α,* which is involved in general up-regulation of protein synthetic capacity (Berberich *et al.*, 1995; Dunn *et al.*, 1993; Hong *et al.*, 1997). Alcohol dehydrogenase is up regulated and may aid in anaerobic metabolism during periods of ice encasement (Jarillo *et al.*, 1993). The levels of cold up-regulated heat shock proteins also increase. These may be used for protein folding and assembly (Parswell and Lindquist, 1993). The heat shock proteins may play roles in protecting proteins from denaturation at low temperature or they may be up regulated to deal with increased demand for protein folding and assembly caused by the up regulation of general protein synthetic machinery during cold acclimation.

Other up regulated genes or proteins probably play protective roles such as sucrose phosphate synthase and $\Delta^{(1)}$pyrroline-5-carboxylate synthase, which are involved in sucrose synthesis and proline synthesis, respectively, and contribute to solute accumulation (Guy *et al.*, 1992b; Reimholz *et al.*, 1997; Igarashi *et al.*, 1997).

A third group of up-regulated genes or proteins include those that are regulatory such as RNA-binding proteins (Carpenter *et al.*, 1994; Dunn *et al.*, 1996; Molina *et al.*, 1997), protein kinases and 14-3-3 proteins (Holoppa and Walker-Simmons, 1995; Hong *et al.*, 1997; Monroy and Dhindsa, 1995; Jarillo *et al.*, 1994), and the *CBF* genes which activate expression of the four cold regulated *COR* genes from *Arabidopsis* and presumably many other genes

associated with increased freezing tolerance (Gilmour *et al.*, 1998; Jaglo-Ottosen *et al.*, 1998).

In addition to the genes whose functions or partial functions are known, many more genes have been identified that encode polypeptides whose functions are not known. Some recent reviews have listed collections of genes with known functions, as well as those with speculative functions and unknown functions (Thomashow, 1999; Pearce, 1999). Many of the polypeptides whose function is not known are either novel or share similarities to LEA proteins. LEA proteins are late embryogenesis abundant and have been found to accumulate during development just prior to the time when seeds undergo dehydration and enter a stress resistant dormant state. Several classes of LEA proteins have been identified and separated into groups based on differences in the types of motifs found in their sequence.

These LEA genes have been isolated from plants of all types and a major group belongs to the dehydrin family (Close *et al.*, 1989). Dehydrins (also called the LEA D-11 or group 2 family of developmentally induced seed proteins) (Baker *et al.*, 1988), can reach levels as high as 1% of total soluble protein during water stress or low temperature stress (Close *et al.*, 1989; Bray, 1993). Some common features among many of these polypeptides are that they are hydrophilic and remain soluble upon boiling. LEA proteins have relatively simple amino acid composition with repeated sequence motifs and regions capable of forming amphipathic α-helices. The dehydrins are recognized by a highly conserved lysine (K) rich sequence, EKKGIMDKIKEKLPG, called the K segment (Close,

1996). The numbers of K segments found in dehydrins vary from one to eleven or more and are predicted to form an amphipathic α-helix which may function to stabilize subcellular structures and membranes (Close, 1996; Dure, 1993). A possible mechanism for protection may be through retention of the interaction between the aqueous cytosolic material, proteins and membranes, and prevention of membrane-membrane contact during periods of low water activity such as that caused by freezing induced dehydration (Close, 1997; Pearce, 1999), which could lead to the types of damage described earlier. A second sequence often found in these proteins is (V/T)DEYGNP known as the Y-segment. Outside of the conserved sequences there is little conservation of sequence between different member proteins in the family. However they frequently contain high content of glycine and other polar amino acids.

In wheat, cold induced members of one family of dehydrins are localized in the cytoplasm and nucleus (Houde *et al.*, 1995). The polypeptide sequence from a member of the family, WCS120, has 6 K-segments and 11 Y-segments and is rich in glycine and threonine. The *WSC120* gene has been shown to be up-regulated by cold within 24 hours and remains at elevated levels while the plant is in the cold, however mRNA levels decrease rapidly during deacclimation (Houde, 1992).

The cold regulated gene *wcor410* is also associated with acclimation to freezing stress in several *gramineae* species (Danyluk *et al.*, 1998). Immunolocalization studies have shown the protein to be closely associated with

the plasma membrane. It has been proposed that WCOR410 helps to prevent membrane destabilization during freeze induced dehydration.

It has not yet been possible to directly test the role of the dehydrins or other LEA proteins in mutant plants with knock-outs in part due to the redundancy in families of *LEA* genes. However, a heterologous expression system has been used to test activity of several isolated LEA proteins. Yeast expressing *Em*, a group 1 wheat LEA (Swire-Clark and Marcotte, 1998), LE25, a tomato group 4 LEA (Imai *et al.*, 1995), LE4, a tomato group 2 and HVA1, a barley group 3 LEA (Zhang *et al.*, 2000), all show improved survival after freezing stress. Generalized roles ascribed to these proteins, based in part on structure and function in the yeast, include ion scavenging and stabilizing membranes and proteins. Continued success with expression of plant genes in yeast could help further dissect the mechanisms of stress tolerance and protection by the different types of stress proteins.

Several other cold-responsive proteins, some of which are classified as LEA or LEA-like and others as novel proteins, are also highly hydrophilic and boiling stable, including the *COR* (*co*ld *r*egulated) gene family of proteins in *Arabidopsis*. At least four groups of *COR* genes have been isolated that show elevated levels of mRNA transcripts within four hours of transfer to low temperature (Thomashow *et al.*, 1990; Gilmour and Thomashow, 1991; Gilmour *et al.*, 1992). The mRNA transcript levels remain high for the duration of low temperature treatments and rapidly decrease upon transfer to warm temperature. Key representatives from each group include *COR6.6*, *COR15a*, *COR47*, and

COR78. The *COR* genes have also been termed *LTI* (low temperature induced), *KIN* (cold inducible), *RD* (responsive to desiccation) and *ERD* (early dehydration-inducible) (Nordin *et al.*, 1993; Wang *et al.*, 1994; Welin *et al.*, 1994; Welin *et al.*, 1995; Wilhelm and Thomashow, 1993; Yamaguchi-Shinozaki and Shinozaki, 1993b). Each of the groups has been found to exist as tandem gene pairs physically linked in the genome and with members of each pair being differentially regulated. At least one member of each pair is induced in response to low temperature. Many of these genes also respond to other treatments associated with water deficit including dehydration, high salt concentrations and ABA.

One of the more thoroughly studied *COR* genes is *COR15a*, which has been shown to play a direct role in freezing tolerance. *COR15a* encodes a 15kDa polypeptide that is targeted to the chloroplast where it is processed to a 9.4kDa polypeptide. Constitutive expression of *COR15a* in transgenic *Arabidopsis* was shown to increase the freezing tolerance of both chloroplasts and isolated leaf protoplasts by 1°C to 2°C. Currently, COR15a is believed to enhance freezing tolerance by helping to stabilize membranes which prevents the formation of or lowers the temperature at which lamellar to hexagonal II phase membrane transition occurs (Steponkus *et al.*, 1998). The improvement in freezing tolerance in the plants overexpressing *COR15a* is subtle since no obvious enhancement is observed at the whole plant level (Artus *et al.*, 1996). Such has also been the case for some other cold-induced genes that have been overexpressed in transgenic plants (Zhu *et al.*, 1996; Kaye *et al.*, 1998).

Additional Low Temperature Induced Genes and Roles in Low Temperature Signal Transduction

The mechanisms by which plants perceive a low temperature signal and how they trigger responses are poorly understood. What many of the components are that make up the earliest steps of the signal transduction pathway are unknown. Sensation of cold may be relayed through changes in membrane physical properties (Nishida and Murata, 1996; Vigh *et al.*, 1993) but direct evidence in higher plants is lacking. Recognition of changes in the redox status of photo-system II has also been suggested as a sensing mechanism for cold (Gray *et al.*, 1997). The role of either in plants is yet to be determined.

Calcium and protein phosphorylation play an important part in the acquisition of freezing tolerance. Calcium may play a role as a second messenger relaying the information of a temperature change. Phosphorylation may then affect the regulation of low temperature signal transduction (Knight *et al.*, 1996; Monroy and Dhindsa, 1995; Monroy *et al.*, 1993; Tahtiharju *et al.*, 1997; Polisensky and Braam, 1996). In *Arabidopsis*, a cold shock elicits an immediate rise in cytosolic free calcium concentrations (Knight *et al.*, 1996; Polisensky and Braam, 1996). This may result from the cold-induced opening of membrane calcium channels or influx from the vacuole (Burk *et al.*, 1976; Monroy and Dhindsa, 1995; Monroy *et al.*, 1993; Knight *et al.*, 1996). Chemical treatments to block calcium channels or inhibit protein kinases severely affect the capacity of plants to develop cold-induced freezing tolerance and limit expression of some cold regulated genes (Knight *et al.*, 1996; Monroy and Dhindsa, 1995;

Monroy *et al.*, 1993; Tahtiharju *et al.*, 1997). However, Monroy and Dhindsa (1995) have shown in alfalfa the addition of a calcium ionophore to cells caused an influx of calcium and induced the expression of two *cas* (cold acclimation specific) genes at 25°C.

Following the increase in calcium flux, transcript levels for several protein kinases have been shown to increase (Jonak *et al.*, 1996; Mizoguchi *et al.*, 1996; Monroy and Dhindsa, 1995; Tahtiharju *et al.*, 1997). In *Arabidopsis* a MAP kinase and MAP kinase kinase kinase are induced in response to low temperature (Mizoguchi *et al.*, 1996). Calcium dependent protein kinases in alfalfa (Monroy and Dhindsa, 1995) and *Arabidopsis* (Tahtiharju *et al.*, 1997) were also activated by low temperature. Application of protein kinase inhibitors blocks the induction of cold acclimation and cold regulated gene expression while addition of a phosphatase inhibitor stimulated the induction of *cas15*. Total phosphatase activity and phosphatase 2A is inhibited by low temperature treatment and 2A is also decreased by calcium ionophore treatment (Monroy *et al.*, 1998), demonstrating a potential link between activation of a calcium cascade, protein phosphorylation and induction of freezing tolerance.

Low temperature stress responsive promoter elements involved in the transcriptional control of a subset of cold upregulated genes are known. Within the promoter of the *Arabidopsis COR* genes is a DNA sequence element, the CRT (C-repeat), which has a core sequence CCGAC that is sufficient for low temperature responsiveness of these genes. It is also found in the wheat *wcs120* and *Brassica napus BN115* low temperature regulated genes and

undoubtedly many others. This core sequence interacts with the C-repeat binding factor (CBF) under different stress conditions and makes up part of a low temperature signal transduction pathway.

CBF is part of a small gene family (*CBF1, CBF3* and *CBF2*; also termed *DREB* for dehydration responsive element binding protein (Liu *et al.*, 1998)) located in direct repeats in the genome. The *CBF*s contain an acidic activation domain, a DNA binding domain motif found in APETALA2 and other plant transcription factors and a possible nuclear localization sequence. CBF1 was first shown to be a functional transcription factor in yeast with its ability to activate reporter genes containing the CRT in the promoter region (Stockinger *et al.*, 1997).

Overexpression of *CBF1* in transgenic *Arabidopsis* upregulates the expression of a family of *COR* genes that have the CRT in their promoter without a low temperature stimulus (Figure 1.1). When freezing tolerance was assayed by an ion leakage test, transgenic plants overexpressing *CBF1* showed a dramatic increase in freezing tolerance over non-transgenic plants that had not been cold acclimated and transgenic plants overexpressing just a single *COR* gene, *COR15a*. At a whole plant level, an increase in freezing tolerance is also observable (Jaglo-Ottosen *et al.*, 1998). These experiments demonstrate that *CBF*-mediated cold induced genes play significant roles in freezing and stress tolerance. The isolation of *CBF* was a major advancement that helped identify an important early component of a low temperature signal transduction pathway and was discovered using a reverse genetic approach. Using repeats of the

CBF

COR

COR

COR

COR

eIF4

Figu
RNA
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Ove
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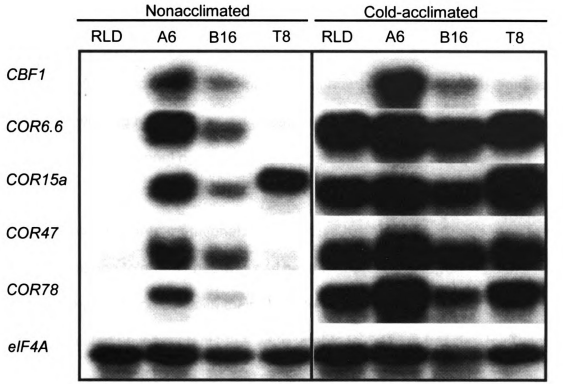

Figure 1.1. Expression of *CBF1* and *COR* genes in *Arabidopsis* plants. Total RNA was prepared from leaves of nonacclimated and 3-day cold acclimated plants and examined for *CBF1* and *COR* gene transcript accumulation by RNA blot hybridization analysis. Amounts of *COR* gene transcripts are detectable in nonacclimated *CBF1* overexpressing plants (A6, B16) at a higher level than nontransgenic plants (RLD). In nonacclimated A6 plants, *COR* transcripts accumulate to levels approximating that seen in cold acclimated plants. Overexpression of *CBF1* did not affect transcript concentrations of *eIF4A* (eukaryotic initiation factor 4A) (Metz et al., 1992), a constitutively expressed gene that is not responsive to low temperature and was used here to compare RNA loading. Transgenic plants overexpressing *COR15a* (T8) only increased transcript accumulation for *COR15a*.

CRT in the promoter of a reporter gene in a yeast one-hybrid screen, Stockinger *et al.*, (1997) was able to identify *CBF1*. Additional links backward in the *COR* gene signaling pathway will help better define the significance of this particular pathway. A more complete understanding of how a low temperature signaling network involving multiple signaling pathways may work will not be possible however until additional components associated with cold acclimation are defined.

Recently, three mutational screens have been used in *Arabidopsis* to help dissect freezing tolerance pathways. The first approach involved isolating mutants that were defective in their ability to cold acclimate. Warren *et al.*, (1996) isolated seven mutants that were unable to develop freezing tolerance even after extended periods of cold acclimation. These mutants were named *sfr* for sensitive to freezing. The reason for sensitivity in some of the *sfr* mutants appeared to be due to an inability to accumulate soluble sugar during cold acclimation (McKown *et al.*, 1996). One of the mutants, *sfr6*, was shown to be deficient in CBF-mediated induction of *COR* genes which confirms the importance of the CBF pathway in cold acclimation (Knight *et al.*, 1999). The additional *sfr* mutants did not affect *COR* gene expression. Most of the *sfr* mutants still retain some capacity to cold acclimate suggesting the mutation blocks one signaling pathway but also indicating involvement of multiple pathways.

A second mutational approach was used to generate plants with aberrant expression of a reporter gene driven by the *COR78* promoter (Ishitani *et al.*,

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1997). Since the *COR78* promoter has cold, drought and ABA responsive elements, this approach allows identification of signal components in all three pathways, at least as they are transmitted through to the *COR* genes. Several hundred mutants with altered reporter gene activity were identified. The mutants fall into three general classes. The *cos* mutants show constitutive expression of osmotically responsive genes. The *los* mutants show loss of expression of these genes, and the *hos* mutants show hyper-expression. Two of the *hos* mutants, *hos1* and *hos2*, are less freezing tolerant after cold acclimation even though they over-accumulate *COR* gene transcripts when exposed to cold (Ishitani *et al.*, 1997, Lee *et al.*, 1999). The *HOS1* locus appears to be a negative regulator of cold signal transduction but may be a positive regulator for other stress signal pathways. *HOS2* also appears to be a negative regulator of cold acclimation through the *COR* pathway but another low temperature requiring process, vernalization, is unaffected. HOS2 may play a positive role in regulation of factors subsequent to *COR* gene expression such as changes in sugar metabolism or membrane composition. Analysis of the different types of mutants isolated by this approach suggest a network of multiple signaling pathways that share components in different pathways to activate the *COR* genes in response to several different stress factors.

A third mutagenesis approach involved the isolation of constitutively freezing tolerant (*cft*) mutants. These mutants are more freezing tolerant than wild type in the absence of cold acclimation. Xin and Browse (1998) isolated twenty-six mutants with increased freezing tolerance. One of the mutants,

eskimo1, tolerates freezing to –10.6°C without cold acclimation due to its high levels of proline and soluble sugars. It does not express the *COR* genes in the absence of cold acclimation. Characterization of additional mutants should provide insights into other aspects of freezing tolerance signaling pathways (Xin and Browse, 2000).

Conclusion

Over the last several years, advances in technology including methods in molecular biology, and the completion of sequencing of the *Arabidopsis* genome and partial sequencing of genomes from several other plants types and the ability to study genome wide expression of genes through micro-arrays has allowed leaps in our understanding of the signaling pathways. A great deal of information however is still lacking and it will be up to those continuing and advancing these technologies to dissect in finer detail the specific mechanisms and interactions involved in signal transduction. Already the picture is complex with the possibility of several different pathways working at the same time to provide freezing tolerance to plants. The reason for the redundancy is not clear but is sure to provide fodder for additional research in the future.

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Chapter 2

INITIAL CHARACTERIZATION OF *CBF* GENE FUNCTION AND IDENTIFICATION OF A PROMOTER REGION SUFFICIENT FOR LOW TEMPERATURE REGULATED EXPRESSION OF *CBF2*

Summary

Arabidopsis COR (cold regulated) genes are induced in response to many different environmental stresses. Promoter analysis of these genes has led to the identification of *cis*-acting elements including a cold and dehydration responsive DNA regulatory element termed the CRT (C-repeat)/DRE (dehydration-responsive element) (Baker *et al.*, 1994; Yamaguchi-Shinozaki and Shinozaki, 1994). Subsequently, a transcription factor that binds to the CRT/DRE was identified and termed CBF (C-repeat binding factor)/DREB (dehydration responsive element binding protein) (Stockinger *et al.*, 1997; Liu *et al.*, 1998). A small family of these transcriptional activators (*CBF1, CBF2*, and *CBF3*) was later identified, all of which bind to the CRT/DRE and activate expression of reporter genes in yeast that contain the CRT/DRE as an upstream activator sequence. In addition, overexpression of *CBF1* and *CBF3* in *Arabidopsis* plants grown at non-acclimating temperatures induces *COR* gene expression and increases freezing tolerance.

In this study, the *CBF* genes and their promoters were further examined to characterize their role in the low temperature signaling cascade and to identify the means for their own regulation. Transcript levels for all three *CBF* genes increased within 15 min of transferring plants to low temperature followed by the

accumulation of *COR* gene transcripts at two to four hours. The *CBF* transcripts also accumulated in response to mechanical agitation, drought, ABA, and cycloheximide. Promoter deletion analysis identified a 155 base pair region that is sufficient for responsiveness to low temperature and the other stresses. The *CBF* genes do not appear to be autoregulated and likely require additional transcriptional regulators, possibly both positive and negative, to mediate their expression.

Introduction

During their life cycle, plants frequently experience many different environmental stresses that can significantly impact their productivity and ability to survive. Because of their immobility, they must make metabolic and structural changes to cope with these environmental conditions. An inducible genetic system responsive to stress stimuli enables many plants to survive. A number of genes that respond at a transcriptional level to stresses such as low temperature, drought and high salinity have been described. The function of a few is known.

In *Arabidopsis*, a group of four sets of gene pairs, each pair represented by a tandem repeat, have been described which are abundantly expressed in response to low temperature. These are termed the *COR* (cold regulated) genes. Transcripts for the *COR* genes begin to accumulate within two to four hours after experiencing cold temperatures. The COR15a protein, the best characterized of the *COR* gene products, has been localized to the stromal compartment of the chloroplast and may play a role in minimizing damage between chloroplast and plasma membranes during freeze thaw cycles

(Steponkus *et al.*, 1998). Functions of the other *COR* genes products are not known. Part of the stress responsive induction of the *Arabidopsis COR* genes is mediated at a transcriptional level (Baker *et al.*, 1994). In fact, the promoters from many stress inducible genes have been analyzed for responsiveness to low temperature, drought and high salinity and different mechanisms for regulation have been proposed (Ishitani *et al.*, 1997). ABA is often associated with stress responses in plants. ABA levels do increase in many plants in response to low temperature (Chen and Gusta, 1983). The *COR* genes, for example, are responsive to applications of ABA (Thomashow, 1994). The cold inducible *Arabidopsis RAB18* gene is also dependent on ABA for activation and the gene is not induced by low temperature in the ABA mutants *aba1* or *abi1* (Lång and Palva, 1992). *RAB18* has an ABA-responsive *cis*-acting DNA sequence element in its promoter that may be responding to the binding of a bZIP type of transcription factor (Guiltinan *et al.*, 1990, Meshi and Iwabuchi 1995, Shen *et al.*, 1996). However, using the same ABA mutants, but this time looking at *COR* gene response, a second pathway was discovered that was not directly dependent upon ABA.

With certain *COR* genes, ABA induced expression is impaired in the ABA mutants, however, cold induced expression is not affected (Gilmour and Thomashow, 1991, Nordin *et al.*, 1991). Subsequent deletion analysis of the promoter from *COR15a* led to the discovery of the *cis*-acting DNA sequence element required for the cold induced expression. This element was termed the CRT (C-repeat) (Baker *et al.*, 1994) and contains the core sequence CCGAC.

This core sequence was also present in a *cis*-acting sequence motif discovered by Yamaguchi-Shinozaki and Shinozaki (1994). Their element was termed the DRE (dehydration responsive element) because it could elicit dehydration-induced expression from genes whose promoters contained this element. The identification of a transcription factor, *CBF1*, which interacts with the CRT/DRE (Stockinger *et al.*, 1997), has advanced our understanding of an important part of the low temperature signal transduction pathway. Liu *et al.*, (1998) subsequently identified the same factor and named it *DREB1b* (*D*ehydration *R*esponsive *E*lement *B*inding protein *1b*). Insights gained from the low temperature stress pathways can aid our understanding of other stress responses and pathways. Ultimately, this may facilitate the development of applications to enhance plant responsiveness to stress. For example, the activation of a cold acclimation response can be achieved by expression of a single transcription factor such as *CBF1*. This gene could have great agronomic potential in crops that are subject to unexpected frost during their growing season. Understanding the basic science behind freezing tolerance and regulation of genes such as *CBF* is also important because the induction of a freezing tolerance state can be costly to a plant. If it were not, evolution might have produced constitutively hardy plants. Such plants would be expected to have a considerable selective advantage in regions or seasons that experience sudden frosts.

In fact, constitutive expression of *CBF*, while it improves freezing tolerance of non-acclimated plants considerably and even improves freezing tolerance of cold acclimated plants (Jaglo-Ottosen *et al.*, 1998), has often resulted in plants

with a retarded growth phenotype (Liu *et al.*, 1998; Gilmour *et al.*, 2000). This has been experimentally overcome in part with the use of an inducible promoter that expresses *CBF* at higher than endogenous levels only after cold treatment. In this example, the *CBF* gene was controlled by the *COR78* promoter, which resulted in hyper-responsiveness to cold and osmotic stress and less growth retardation (Kasuga *et al.*, 1999). Identification of additional factors responsible for regulation may afford a fine-tuning of the expression levels for some of these transcription factors to prevent the negative results that are sometimes seen.

Subsequent to the initial identification of *CBF1*, members of the *CBF* family (*CBF1, CBF2*, and *CBF3,* also termed *DREB1b, DREB1c* and *DREB1a,* respectively, (Liu *et al.,* 1998)) were shown to be transcriptionally regulated. Promoter/reporter fusions and transgenic plants were used to demonstrate low temperature induction. In addition, Gilmour *et al.*, (1998) demonstrated that the *CBF* genes were responsive to mechanical agitation but were unable to determine responsiveness to drought and ABA. However Liu *et al.*, 1998, and Shinwari *et al.*, 1998 suggested they were not induced by drought nor treatments with ABA.

In this study, analysis of expression patterns for the *CBF* genes was performed in order to determine the role of *CBF* in the low temperature signal transduction cascade and to examine the responses of the *CBF* gene promoters to various stimuli. Activity of the *CBF2* promoter was further examined with deletion and nucleotide base substitution experiments in order to determine DNA

sequences that may be important for its responsiveness as well as that of the other *CBF* genes.

Results

Mapping of *CBF* and *COR* genes. Analysis of DNA blot hybridization and subsequent sequencing of a clone from a genomic library screen indicated three *CBF* genes were physically linked in direct repeats in the *Arabidopsis* genome (Gilmour *et al.*, 1998). To determine the map position of the *CBF* gene cluster and that of four CRT-containing *COR* genes, *COR6.6*, *COR15a*, *COR47*, and *COR78*, cosegregation analysis of molecular markers from the Lister and Dean recombinant inbred lines (Lister and Dean, 1993) was conducted. The results indicated that the *CBF* genes were located on chromosome 4 at 75.6cM. The four *COR* genes were distributed throughout the genome unlinked to any other *COR* gene or the *CBF* genes. *COR6.6* was located on chromosome 5 at 32.1cM, *COR 15a* was on chromosome 2 at 76.9cM, *COR47* was on chromosome 1 at 27.4cM and *COR78* was on chromosome 5 at 106.5cM.

Expression of *CBF* genes. The *CBF* genes have been shown to activate expression of a reporter gene carrying a CRT element in its promoter in yeast (Stockinger *et al.*, 1997, Gilmour et at., 1998). Whether the *CBF* genes themselves were constitutively expressed or induced in response to low temperature was another question. A constitutively expressed gene would suggest that the CBF protein was activated in response to low temperature while low temperature induced accumulation of gene transcripts would suggest either a transcriptionally regulated component or an mRNA stability factor. To partially

answer the question, *Arabidopsis* plants were grown at nonacclimating conditions and then transferred to low temperature for various periods of time. RNA was then extracted and blot hybridization was performed using a cDNA for the entire *CBF1* coding sequence, which hybridizes to all three *CBF* genes. The results indicated the *CBF* transcripts increased rapidly within 10-15 minutes after transfer to low temperature. The transcripts continued to increase until about 2 hours of low temperature treatment, then decreased to a new lower level over the 24-hour period of the experiment (Figure 2.1A). Between 2-4 hours after the induction of *CBF*, transcripts for the *COR* genes began to accumulate.

To test whether elevation of transcript levels was due to dehydration stress, we used cultured *Arabidopsis* cells that were suspended in an aqueous medium and thus were constantly hydrated. Transcript levels for the *CBF* and *COR* genes were examined and also found to be elevated in this liquid cell suspension when treated with low temperature (Figure 2.1B).

The CRT/DRE DNA regulatory element found in the promoter of *COR* genes stimulates gene expression in response to both low temperature and dehydration stresses but not to ABA (Yamaguchi-Shinozaki and Shinozaki, 1994). To determine if the *CBF* promoters might also be responsive to these treatments, RNA blot hybridization analysis of treated samples were examined. When using the entire *CBF1* cDNA as a hybridization probe, the apparent *CBF* transcript levels increased within 15 minutes of placing detached leaves on dry filter paper or after spraying plants with a solution of ABA (100μM). The levels of transcript accumulated to a peak at about 30 minutes and then decreased to a

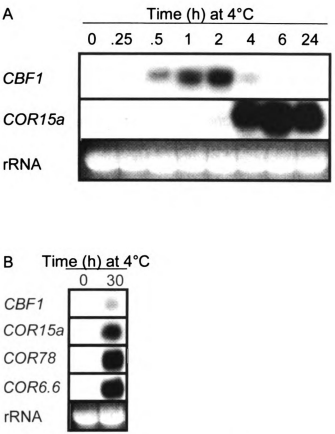

Figure 2.1. Expression pattern of *CBF* and *COR* genes after exposure to low temperature. In A, plants were exposed to low temperature for between 0h and 24h. After treatment, transcript accumulation for the indicated genes was detected by RNA blot hybridization. In B, suspension cultured cells were treated for 30h at low temperature and transcript accumulation was detected for the indicated genes. The rRNA panel shows an ethidium bromide stained gel picture of ribosomal RNA representative of the loading for the blots.

level similar to untreated plants by 24h for dehydration treatments or 4h for ABA treatments (Figure 2.2). These results initially appeared to indicate that *CBF* might be responsive to both ABA and dehydration. However, control experiments in which leaves were detached under water to prevent dehydration or/and in the air and then placed onto wet filter paper or plants sprayed only with water had a similar pattern of expression as the experimental treatments. This suggested that much of the induction seen in the dehydration and ABA test

treatments might actually have been due to another stress such as the mechanical agitation associated with the treatment.

The effects of mechanical stress were tested by growing seedlings in covered Petri dishes, to prevent changes in environmental water status, and then tapping the plate on its side for five minutes to produce a mechanical agitation. RNA blot hybridization was then performed and *CBF* transcripts were observed to increase within 15 minutes and then subside to near untreated levels within an hour after the tapping treatment (Figure 2.2). *COR15a* gene expression also rose in all of the experiments within 2-4h. The increase in *COR15a* expression is presumably due to the large increase in *CBF* expression that resulted from the mechanical agitation stress applied to the plants, however, additional regulatory factors may also be involved. In the dehydration and ABA treatments, *COR15a* transcript remained higher than controls at 24h possibly due to an increase in endogenously produced ABA, which induced *COR15a* expression through the ABREs present in the promoter of the gene (Baker *et al.*, 1994).

Further studies on *CBF* gene expression. Many previous experiments used to examine the expression of the *CBF* genes were carried out only after directly shifting plants from a warm growth chamber (~20°C) to cold (4°C) (Gilmour *et al.*, 1998). Therefore, the question arose whether there was a need for this large temperature change to activate the *CBF* low temperature response pathway. This, in fact, does not appear to be the case (Figure 2.3). Plants that were slowly cooled at a rate of 2°C per hour over an 8h period and then held at 4°C for an additional 16h began to accumulate *CBF* gene transcripts at about 3h

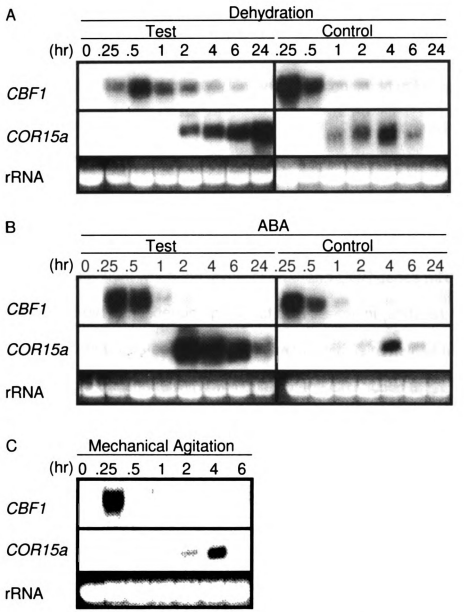

Figure 2.2. Pattern of gene transcript accumulation in response to dehydration, ABA, and mechanical agitation. *Arabidopsis* seedlings were grown on agar medium and then treated as described in the text. Levels of *CBF1* and *COR15a* transcripts were determined by RNA blot analysis. All zero time samples are of control plant material collected prior to treatment. (A) RNA isolated from detached leaves incubated on dry filter paper (Test) or wet filter paper (Control) for the indicated times. (B) RNA isolated from seedlings sprayed with a solution containing ABA (Test) or without ABA (Control). The samples are from times after spray treatment. (C) RNA isolated from seedlings mechanically agitated for 5 min and then incubated for the indicated times with no agitation. The rRNA panel shows an ethidium bromide stained gel picture of ribosomal RNA representative of the loading for the blots.

after the start of the experiment. The ambient temperature surrounding the plant leaves at the point *CBF* mRNA was first visible by RNA blot hybridization was about 14°C. The amount of *CBF* transcripts continued to increase and reached a maximum at 4°C, similar to the amount of transcript accumulation for plants that experienced a cold shock. The amount of *CBF* transcripts after about 10 hours (about 2-3 hours after reaching 4°C) began to decrease to a new steady state level (Figure 2.3).

In addition, when the levels of *CBF* and *COR* genes mRNA were measured over an extended period of cold treatment (21days), this new lower level for *CBF* persisted while *COR* gene transcripts remained high. Plants containing a transgene with two copies of the CRT fused to *GUS* also maintained a high level of transcript throughout the 21-day low temperature treatment (Figure 2.4). This suggested that the lower level of *CBF* transcript was sufficient to maintain the high level of *COR* gene expression. Alternatively, increased stability of the CBF protein during the long period of cold may decrease the need for a high level of *CBF* transcript in order to maintain elevated levels of *COR* gene or *GUS* marker gene transcripts.

Examination of *CBF* promoters for low temperature responsive elements. Gilmour *et al.*, (1998) demonstrated that the *CBF* genes are not autoregulated, and examination of the three *CBF* promoter sequences revealed no regions containing the core pentamer sequence of the CRT/DRE (CCGAC). However, RNA gel blot analysis using *CBF1, CBF2*, and *CBF3* gene specific

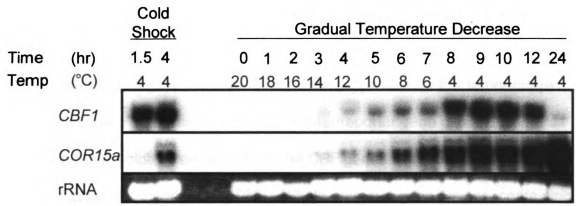

Figure 2.3. Expression pattern of *CBF* and *COR15a* genes after cold shock and a slow temperature downshift. Plants grown at 20°C were transferred directly to a 4°C chamber for cold shock or cooled slowly (0.5°C per 15 min) and sampled at the indicated times. RNA blot hybridization was used to detect transcripts at the sampled time points using a probe for *COR15a* and a full length *CBF1* probe. The rRNA panel shows an ethidium bromide stained gel picture of ribosomal RNA representative of the loading for the blots.

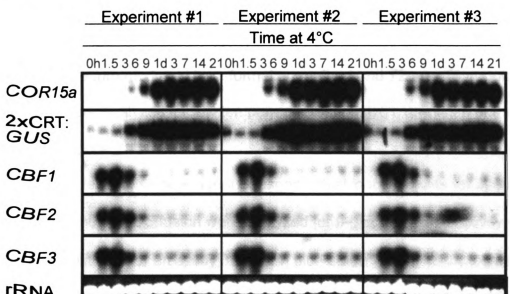

Figure 2.4. Expression of *CBF* genes and their targets after an extended period of cold. Three independent sets of plants containing two copies of the CRT in a chimeric promoter fused to *GUS* (2xCRT:*GUS*) were grown at 22°C before transfer to 4°C for 21 days. RNA was isolated within hours (h) or days (d) after transfer to 4°C. RNA blots were then probed to determine expression of the endogenous *COR15a*, the *GUS* marker and the three *CBF* genes using gene specific probes. The rRNA panel shows an ethidium bromide stained gel picture of ribosomal RNA representative of the loading for the blots.

probes indicated that all three genes were rapidly induced by exposure of plants to low temperatures (4°C) (Gilmour *et al.*, 1998). To investigate the hypothesis that a *cis*-acting element in the promoter was responsible for low temperature responsive gene expression, a set of promoter/reporter gene plasmid chimeras consisting of promoter fragments from the *CBF* genes were fused to the *GUS* reporter gene (Jefferson *et al.*, 1986). Whole promoter gene fusions, containing approximately 1kb of promoter sequence upstream of the translational start site, were created for all three *CBF* genes. Six gene fusions with the 5' end of the *CBF2* promoter deleted and three gene fusions with the 3' end of the *CBF2* promoter deleted were also created (Figure 2.5). The sequence and relevant information about the region of the *CBF2* promoter used in the deletion studies is shown in Figure 2.6. *Arabidopsis thaliana* plants were then transformed by an *Agrobacterium* mediated transformation method and T2 and T3 populations of plants were analyzed for low temperature responsive expression of the *GUS* reporter gene.

At least ten independent transgenic *Arabidopsis* plants for each whole *CBF* promoter *GUS* fusion were analyzed for β-glucuronidase activity which when positive after an assay, results in blue staining in tissues (Jefferson, 1987). After both warm and low temperature treatments, staining was observed in tissues of the plants (Figure 2.7). This seemed to contradict earlier results from RNA blots indicating the endogenous *CBF* gene transcripts were not elevated at warm temperatures. Therefore, RNA blots were prepared for the transgenic reporter lines and hybridized with the *GUS* gene as a probe. For all transgenic

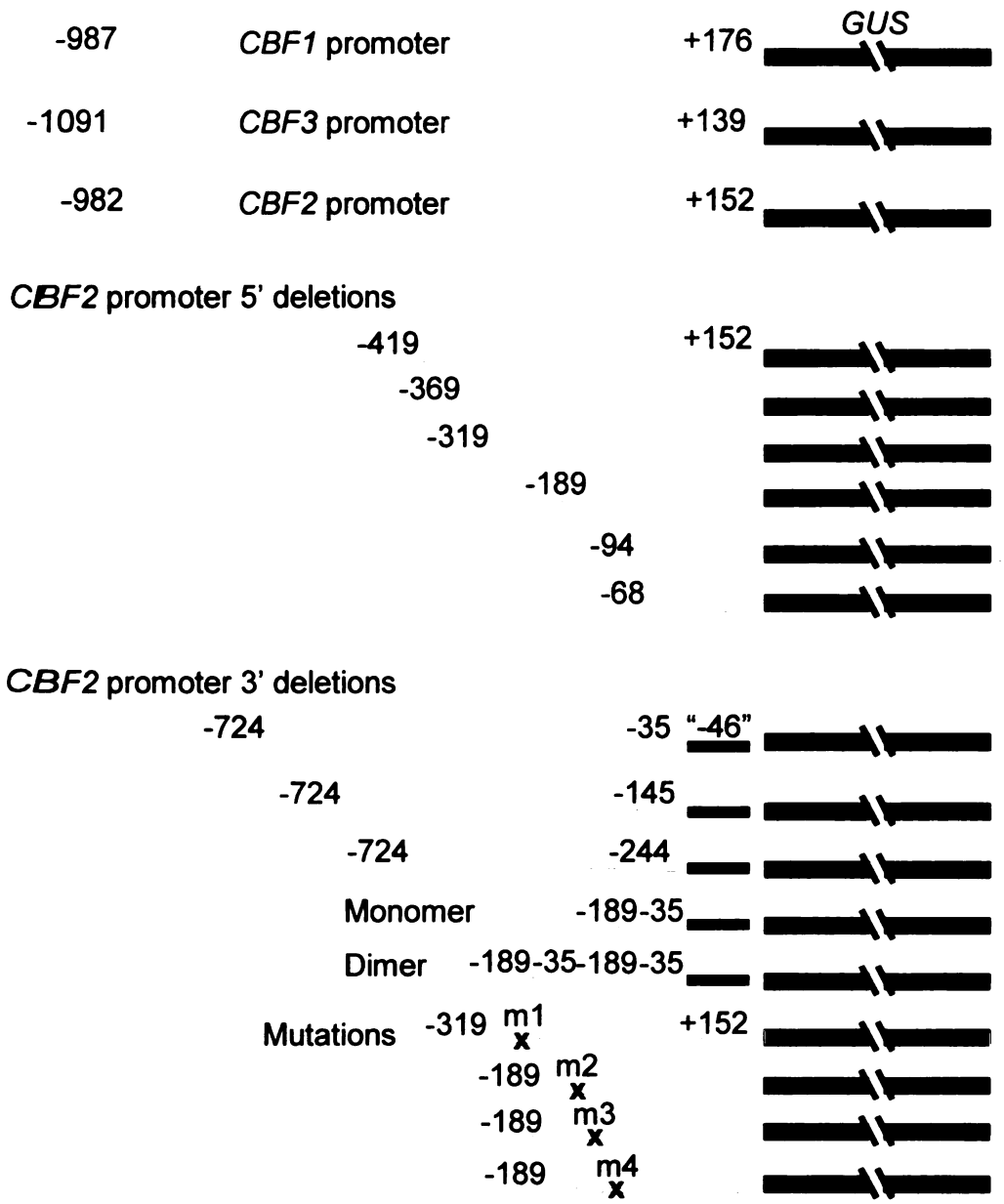

Figure 2.5. Diagram depicting the three *CBF* promoters and *CBF2* promoter fragments used in promoter analysis experiments. Each fragment was inserted into a binary vector upstream of the *GUS* gene. Fragments that had 3' deletions were first inserted into a binary vector upstream of the cauliflower mosaic virus 35S minimal promoter (-46 minimal promoter) then fused to *GUS*.

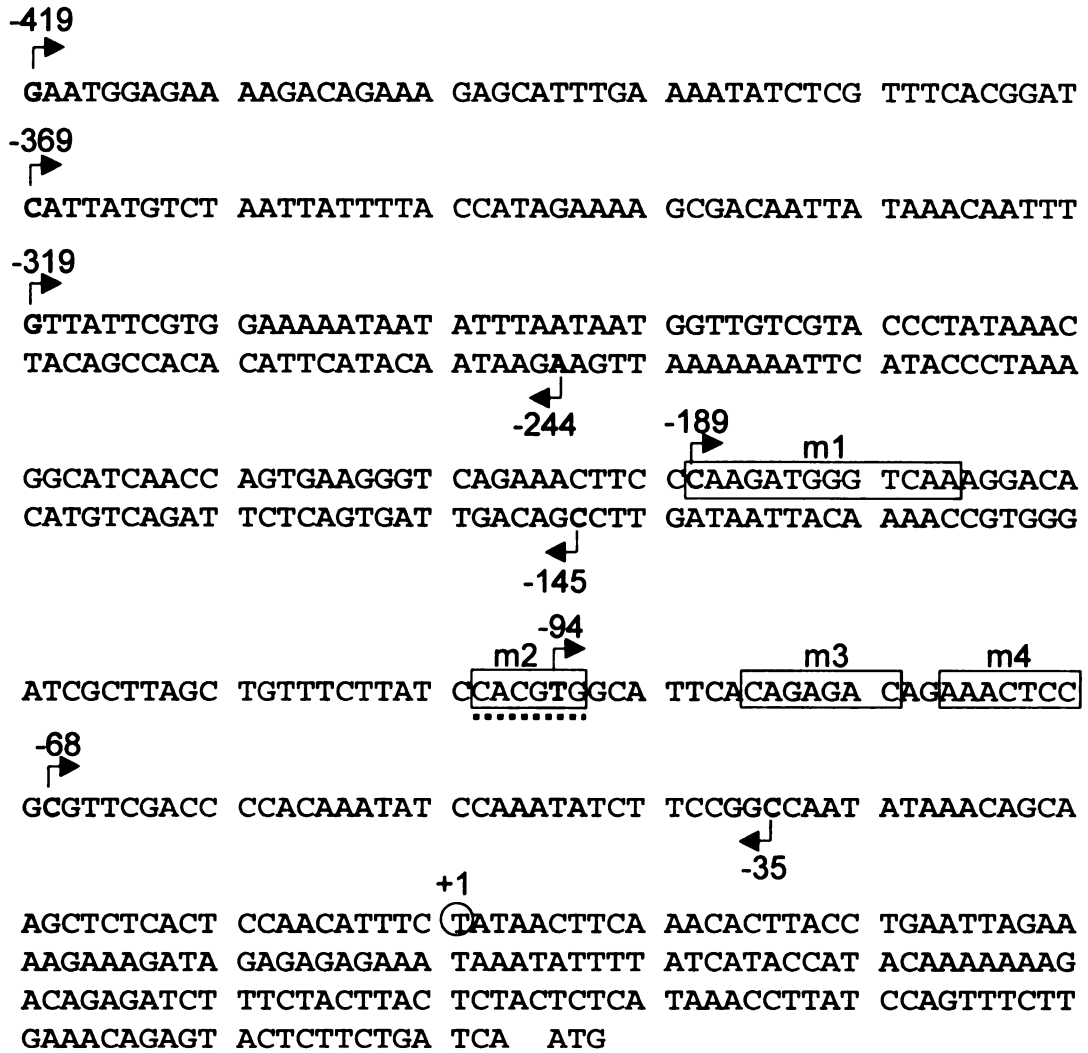

Figure 2.6. Sequence from *CBF2* promoter deletions and mutations from -419 to +152. The +1 indicates the transcription start site. Arrows are used to designate the points of the 5' and 3' deletions. Mutations (m1, m2, m3, and m4) are boxed and were created by deleting the boxed bases and replacing them with a restriction site (Xba I, TCTAGA). A putative ABA responsive element (ABRE) is underlined with a dashed line.

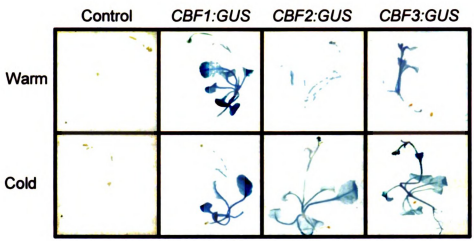

Figure 2.7. Staining of transgenic plants containing whole *CBF* promoter/*GUS* fusions. Representative plants containing promoter fusions from *CBF1*, *CBF2*, and *CBF3* are shown after growth at warm temperatures and after 3 days of low temperature (4°C) treatment. Blue color is seen in the leaves and stems of the plants and indicates β-glucuronidase (GUS) activity resulting from the *CBF* promoter activated *GUS* expression. The control contains the *GUS* gene but no active promoter and therefore no blue color.

lines containing the whole promoter *CBF/GUS* fusion, the *GUS* reporter was induced to high levels by low temperature and was not present or at very low levels at warm temperatures in unstressed plants (Figure 2.8). This suggested that the original staining information from warm grown plants may have resulted from a transiently stress activated promoter or/and developmentally or circadian rhythm induced expression and stability of the *GUS* protein. In fact β-glucuronidase activity resulting from *GUS* gene activation was visible by staining warm grown seedlings within a few days after germination. Seedlings continued

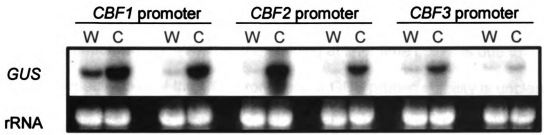

Figure 2.8. Whole promoter *CBF/GUS* fusions demonstrate low temperature induced promoter activity. All three *CBF* promoters accumulated little or no transcript under warm growth conditions, (W), but had increased levels of *GUS* marker transcript within 90 minutes of low temperature treatment, (C), as determined by RNA blot hybridization. The rRNA panel shows an ethidium bromide stained gel picture of ribosomal RNA representative of the loading for the blots.

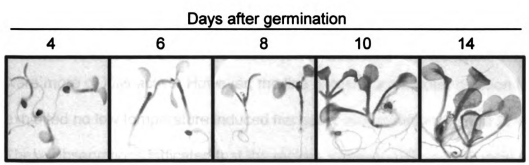

Figure 2.9. Seedling staining indicating *CBF* promoter/*GUS* gene activity. Transgenic plants containing a *CBF2* promoter/*GUS* transgene were monitored for GUS activity at various time points during development at warm temperatures. By 4 days after germination, blue staining was clearly visible in the hypocotyls. Between 8 and 10 days after germination cotyledons and true leaves were also staining blue.

to stain blue at warm temperatures for at least 14 days (Figure 2.9. Charles Herman, unpublished). Whether the staining at later time points is due to continuing activation of the *CBF* promoters or GUS protein stability is unclear. Certainly the *CBF* gene mRNA transcript is not present at the times used in RNA blots and so the disparity may simply be due to GUS protein stability.

Since all three *CBF* promoters seemed to be functioning similarly at an RNA level in regards to low temperature induced expression, only the promoter from the gene *CBF2* was chosen for further deletion analysis. For 5' deletion promoter fusions, the transformants containing deletions to -419, -369, -319, and -189 relative to the presumptive start of transcription showed similar levels of induction (Figure 2.10). The deletion to -94 had elevated transcript levels at low temperature in several lines. However, accumulation was much lower than the longer promoter fusions. Variation in expression levels between lines of a single deletion type may have been caused by populations that were not homozygous for the transgene or by insertion of a transgene in regions within the genome that were more or less active. However, the line carrying a promoter deletion to -68 exhibited no low temperature induced transcript accumulation (Figure 2.10). These observations indicated that the region between -189 and -68 included a *cis*-acting element(s) involved in the low temperature induced expression of *CBF2*.

The 3' deletion lines were then analyzed. The transformants containing the *CBF2* promoter deleted 3' to -35 relative to the start of transcription demonstrated low temperature induced transcript accumulation. The

transformants containing a *CBF2* promoter fragment deleted to -244 or -145 showed very low levels of induction or even loss of low temperature induced transcript accumulation (Figure 2.10). This again indicated loss of an element(s) necessary for promoter activity between -145 and -35 and also that the apparently inducible expression was not due to a transcript stability function associated with the 5' untranslated region since this region is not present in the 3'deletion plasmids.

Based on this information, a plasmid was constructed which contained the promoter fragment from -189 to -35 fused to *GUS*. A tandem repeat or dimer of this fragment was also fused to *GUS*. Both plasmids were independently transformed into *Arabidopsis* plants. For each plasmid construct, RNA blots were examined for expression of *GUS* reporter transcripts from several lines of plants. While a low level of expression was seen for the monomer construct, strong induction was observed when the dimer construct was transformed into plants (Figure 2.11). This confirmed the notion of a low temperature responsive element(s) located in this 155 base pair region of the *CBF2* promoter.

Expression of *CBF* genes in response to mechanical agitation, ABA, dehydration, osmotic stress and cycloheximide.

Earlier work had shown the *CBF* genes to be exquisitely sensitive to mechanical agitation, which resulted in abundant transcript accumulation. This response affected our ability to monitor the activity of *CBF* in plants that were subjected to ABA treatments or drought stress because a confounding mechanical agitation response precipitated by the method of treatment obscured

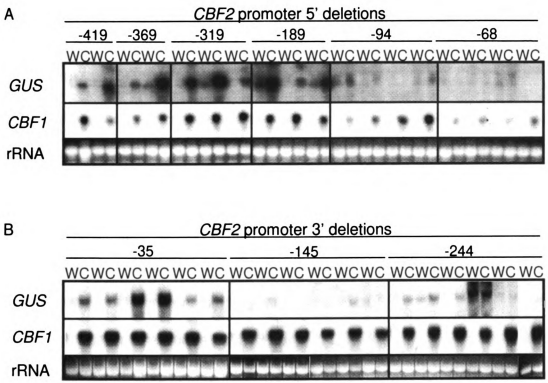

Figure 2.10. Expression pattern of deleted *CBF* promoter/*GUS* fusions. Results from an RNA blot hybridization showing expression of genes from transgenic plants containing the *CBF2* promoter deleted 5' (A) or 3' (B) to indicated lengths and fused to the *GUS* gene. The level of *GUS* and *CBF1* transcript after warm (W) or cold (C) treatment is shown. Each W/C pair represents an independent transgenic line. The rRNA panel shows an ethidium bromide stained gel picture of ribosomal RNA representative of the loading for the blots.

the response. The ABA response may also have been obscured by an osmotic stress response since plants were treated with a hypotonic solution with or without ABA. Subsequently, procedures were developed that minimized mechanical stimulation and osmotic stress and enhanced the visualization of transcripts so that the effects of ABA and drought stresses on *CBF* expression could be better observed. Plants containing whole promoter *GUS* fusion were grown on agar medium and treated with 100μM ABA over a 24h period, or

dehydrated over a 6h period, and expression of the endogenous *CBF* genes and the *GUS* reporter were observed. All three genes and the *GUS* gene fused to the *CBF* promoters responded to both ABA and dehydration treatments (Figure 2.12). While the transcript levels due decrease rapidly after ABA treatment, they initially appear higher and remain higher than controls for about two hours. The decrease in transcript may reflect a decrease in active ABA levels as light has a tendency to racemerize the ABA to an inactive form. Alternatively the initially high levels of transcript in ABA treatment samples may be a result of an augmentation of the mechanical agitation response and the presence of transcript at later time points simple reflects decay from a higher level. This alternative does not appear to be the case for drought treatments since transcript levels are maintained through the treatment time and are not maintained in the controls. Endogenous *CBF* transcripts were not induced to as high a level as when the plants experienced a cold stress. This could result if rapid turnover of the *CBF* RNA was retarded by low temperature. In fact turnover rate of the *CBF* transcript was very rapid. Within 90 minutes of low temperature treatment, *CBF* transcript accumulation reached a peak but in less than 90 minutes after stopping treatment, transcript was no longer visible by RNA blot hybridization (Figure 2.13). The turnover rate for *COR15a* was less than 8 hours (Hajela, *et al.*, 1990) but is not visible in figure 2.13 because sample times were not extended beyond 90 minutes. Additional evidence suggesting rapid turnover of *CBF* transcripts and a comparison of the relative activity of a *CBF* promoter after cold, ABA and drought treatment is shown in Figure 2.14. The relative level of endogenous

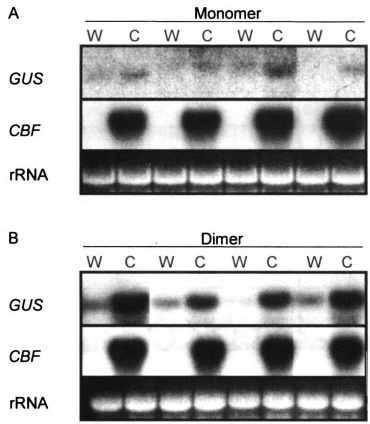

Figure 2.11. Monomer and dimer promoter fragment expression patterns. Expression of the *GUS* transgene was examined in transgenic plants containing the monomer 155bp fragment fused to *GUS* and the dimer of the 155bp fragment fused to *GUS*. Expression from the endogenous *CBF* gene is shown as well. Each pair of lanes (W= warm, C= cold) represents an independently transformed plant line. The rRNA panel shows an ethidium bromide stained gel picture of ribosomal RNA representative of the loading for the blots.

CBF transcript is high at cold temperature but low within 2h after treatment with ABA or drought. The level of *GUS* transcript when fused to the *CBF2* promoter however, is relatively high for ABA treatment but less so for drought. The difference between the level of transcripts for figure 2.12 and 2.14 are due in part to the length of time the blots was exposed to film. Blots from figure 2.12 were exposed over three times longer to help visualize the transcripts.

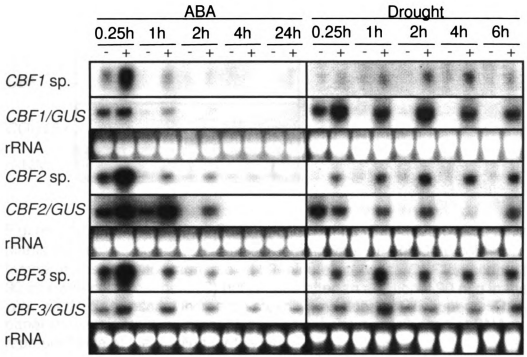

Figure 2.12. Effects of ABA and drought treatments on *CBF* expression. RNA blot hybridization of *CBF*promoter/*GUS* fusions after treatment with ABA or drought stress for indicated times. Each blot row pair represents a different *CBF* gene promoter fused to *GUS* and probed with either a gene specific probe (sp.) or a *GUS* probe. ABA treatment required floating plants on water (-) and a solution with ABA (+). Drought treatment included dehydrating whole plants in a desiccator (+) while controls (-) were handled the same as drought stressed plants but kept sealed in Petri dishes. Hybridization blots were exposed for ten days to visualize bands. The rRNA panel shows an ethidium bromide stained gel picture of ribosomal RNA representative of the loading for the blots.

An osmotic stress response as a result of treating plants with a hypotonic solution during ABA experiments was also observed. Warm grown plants transferred directly to water often had higher "background" levels of *CBF* transcripts present when compared to plants that were transferred to liquid growth medium. Figure 2.15 demonstrates a dramatic example of this. The level of background transcript present when plants were placed in water was not

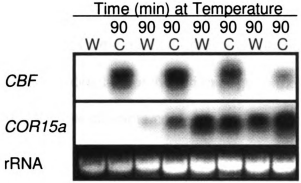

Figure 2.13. Turnover rate of *CBF* gene transcript. Rapid turnover is seen when plants are repeatedly moved from warm temperatures (24°C) to cold temperatures (4°C) to warm, etc. at 90-minute intervals. *CBF* transcripts peak by 90 min with cold treatment (C) but is gone after an additional 90 min in the warm (W). *COR15a* transcript increases steadily and does not disappear. The rRNA panel shows an ethidium bromide stained gel picture of ribosomal RNA representative of the loading for the blots.

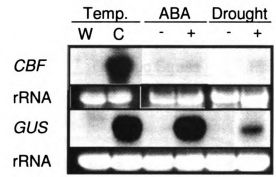

Figure 2.14. Relative promoter activity after cold, ABA and drought treatments. Endogenous *CBF* gene transcript levels were determined for warm grown (W) and 1.5h cold (C) treated plants as well as for plants treated for 1.5h with ABA or drought stress. (-)= control for each treatment, and (+) = treatment condition. The level of *GUS* transcript in transgenic plants containing a *CBF2* promoter fused to *GUS* was also measured using similar treatments. Hybridization blots were exposed to film for three days. The rRNA panel shows an ethidium bromide stained gel picture of ribosomal RNA representative of the loading for the blots.

always this high.

To investigate whether a *cis*-acting element in the promoter of *CBF2* was also responsible for induction of gene expression by mechanical agitation, the same lines of plants containing *CBF2* promoter *GUS* fusions were treated by mechanically agitating seedlings on plates. Significant levels of transcript accumulation had been observed within 15 minutes of treatment for whole promoter *GUS* fusions (Figure 2.16A). In fact, the peak level of expression resulting from continuous mechanical agitation appeared to occur within 15 min to 1 h and then quickly decreased (Figure 2.16B). To test for responsiveness to mechanical stress in the promoter deletion lines, plants were agitated for 15 minutes and transcript accumulation was observed. In response to mechanical stress, 5' deletions to -419, -369, -319, and -189 had increased transcripts. A much lower level of transcript accumulated for the 5' deletion to -94, while loss of

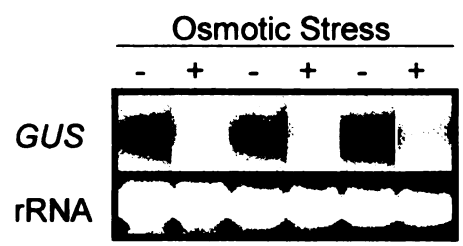

Figure 2.15. Response of *CBF* promoter to hypo-osmotic shock. Three independent lines of transgenic plants containing the *CBF2* promoter fused to *GUS* were treated by transferring them from solid medium to plates containing either distilled water (-) or liquid growth medium (+). Transcript levels for *GUS* were then examined. The rRNA panel shows an ethidium bromide stained gel picture of ribosomal RNA representative of the loading for the blots.

expression was observed in the plants containing a transgene with a promoter deletion to -68 (Figure 2.16C). The plants containing promoter/marker fusions with 3' deletions to -35 had mechanical stress induced transcript accumulation but those with promoter fragments deleted to -294 and -145 showed no activity. The 155 base pair fragment as a monomer and dimer was also checked and transgenic plants containing them also showed elevated expression levels of the marker for both with much higher levels for the dimer (Figure 2.16C). These patterns of expression were seen in at least two independent lines for each construct.

Finally the accumulation of *CBF* was measured in response to the addition of cycloheximide. Seedlings containing the 155 base pair fragment as a dimer were treated for 90 minutes at room temperature after addition of cycloheximide to the Petri dishes. RNA hybridization using the *GUS* probe demonstrated an increase in *CBF* transcript while no such increase was seen in controls without cycloheximide but handled identically (Figure 2.17).

Mutations in the promoter. Within the 3' region of the *CBF2* promoter that is sufficient for low temperature responsive gene expression (-189 to -35, Figure 2.6), there is a DNA sequence element that resembles an ABA responsive element (ABRE). Site directed mutagenesis was used to alter this sequence to determine if it played a role in the stress response. The mutated promoter fragment responded to treatment with ABA similar to non-mutated controls (Figure 2.18A). Three additional mutations were created elsewhere in the promoter where conservation of sequence was found between the other three

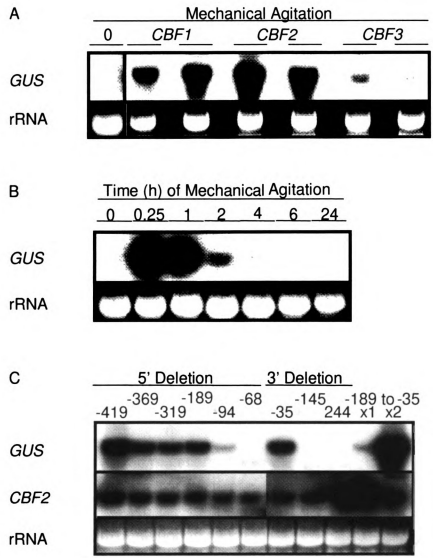

Figure 2.16. Responsiveness of *CBF* promoters to mechanical agitation. In A, an RNA blot hybridization shows the response of whole *CBF* promoters fused to *GUS* and treated by shaking seedlings, that had been grown in sealed Petri dishes, for 15 minutes. The 0 lane is a control taken before plants were shaken. Each lane represents an independently transformed plant containing the promoter/*GUS* fusion (two lines for each promoter/*GUS* fusion). In B, the result from continuous mechanical agitation over 24 hours is shown for the *CBF2* promoter/*GUS* fusion. In C, plants containing promoter fusions with either 5' or 3' deletions and the monomer and dimer fragments (-189 to –35 x1, x2) were shaken for 15 minutes and RNA hybridized to either the marker *GUS* or the endogenous *CBF2* gene. Similar results were seen when additional lines were tested. The rRNA panel shows an ethidium bromide stained gel picture of ribosomal RNA representative of the loading for the blots.

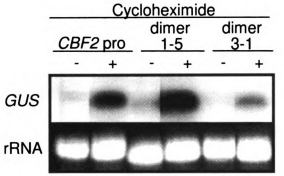

Figure 2.17. Effects of cycloheximide treatment. Warm grown plants containing either the whole *CBF2* promoter or the dimer fragment were treated with cycloheximide (+). Blot hybridization with a *GUS* probe was used to determine the amount of transcript that accumulated in response to the treatment. Control plants were treated in a solution without cycloheximide (-). A plant line containing the whole promoter from *CBF2* fused to *GUS* is compared with two independently generated lines containing a dimer of the 155bp *CBF2* promoter fragment. The rRNA panel shows an ethidium bromide stained gel picture of ribosomal RNA representative of the loading for the blots.

CBF genes. All the mutations were then tested for responsiveness to low temperature (Figure 2.6 and 2.18B). The genes with promoters containing mutations at m1, m2 and m3 were induced to relatively high levels in response to low temperature. However, only marginal levels of induction were seen for many lines containing the mutation at m4. In addition, in some plants the level of transcript present at warm temperatures was almost as high at that seen in the cold.

Discussion

The discovery of *CBF1* with its ability to moderate the cold acclimation process in plants by regulating expression of multiple genes involved in freezing tolerance

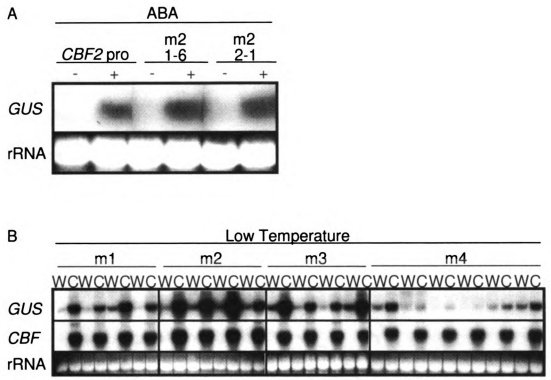

Figure 2.18. Effect of site directed mutagenesis on ABA and low temperature responsive expression. In A, the promoter activity levels from ABA treated plants (+) were determined by blot hybridization using the marker gene *GUS* as a probe. Control plants were treated in a solution without ABA (-). A plant line containing the whole promoter from *CBF2* fused to *GUS* is compared with two independently generated lines containing the *CBF2* promoter fragment deleted to -189 and mutated to remove a potential ABRE (m2). In B, plants containing promoters with site directed mutations were treated with cold (C), or left untreated (W) and expression of *GUS* and *CBF* were compared using an RNA blot hybridization. Each pair of lanes represents independently transformed plant lines. The rRNA panel shows an ethidium bromide stained gel picture of ribosomal RNA representative of the loading for the blots.

was a major advance that has led to a better understanding of some of the early steps in one low temperature signaling cascade. The *CBF1* gene and its two tandem homologs, *CBF2* and *CBF3* are all cold induced (Gilmour *et al.*, 1998). Here the transcript levels for all three *CBF* genes have been shown to increase dramatically within 15 min of transferring plants to a low temperature

environment. Following at about 2-4h after *CBF* induction, the level of transcript for the CRT/DRE containing *COR* genes increases. The kinetics of these experiments suggest that low temperature stimulated increases of CRT/DRE containing *COR* gene transcripts requires a signaling cascade in which *CBF* transcript accumulation is one of the earliest events. Induction does not necessarily require a large change in temperature as was demonstrated when plants were slowly cooled over an 8h period. However, lower temperatures still seemed to stimulate higher levels of transcript accumulation. This could be due to a change in temperature that is still too rapid for the plant to acclimate completely. By 24h the level of *CBF* transcript reaches what appears to be a new steady state level, and this level persists indefinitely if the plant perceives a continuous cold stimulus. If the plant desensitized to the cold stimulus, one might expect the signal to eventually disappear. This is not the case. In fact, the plants respond quite rapidly and to a high level when exposed repeatedly to low temperatures.

CBF induction appears to be regulated at a transcriptional level as was shown when the *CBF* promoters were fused to the *GUS* reporter gene. The putative transcription factors involved in *CBF* induction though are probably not the CBF proteins themselves as the promoters of all the *CBF* genes lack the CRT/DRE sequence and overexpression of *CBF1* does not cause the accumulation of *CBF3* transcript (Gilmour *et al.*, 1998). There are however several conserved motifs in the promoters of all three *CBF* genes and while most do not seem to be required for low-temperature induction, there is still the

possibility they may function to confer specificity or alter responsiveness under certain conditions.

In this study, the three *CBF* promoters in whole and multiple deletion fragments of *CBF2* fused to *GUS* were used to characterize responsiveness to different stresses. While the picture is still not clear as to what minimum element(s) of the promoter is required to induce expression under all conditions, some parts of a 155 base pair region appear to be sufficient. This region was responsive to all the stresses tested including low temperature and mechanical agitation and to a lesser extent ABA and water stress. ABA and dehydration treatments result in distinct patters of expression with drought stress presenting a sustained elevated level of transcript while the ABA treatment resulted in a higher initial level but rapid decay. While this could represent inactivation of ABA in the light, it may also represent an augmentation of the response to mechanical stress which then quickly disappears. In either case, because the responses to ABA and dehydration stresses were low they may have been missed in earlier reports (Liu *et al.*, 1998, Shinwari *et al.*, 1998) due to a relatively large induction caused by mechanical agitation whether intentional or accidental. This difference in the level of expression between low temperature and dehydration or ABA response may mark a bifurcation in the signaling cascade between the two stress responses. The *CBF* genes are mainly responsive to low temperature stress and two additional CRT/DRE binding proteins, *DREB2A* and *DREB2B* (dehydration responsive element binding proteins) identified by Liu *et al.* (1998) are responsive to dehydration stress.

At present the receptor of the low temperature signal (or drought or mechanical signal) is not known nor are the earliest events in the signaling cascade that precede *CBF* induction. Gilmour *et al.* (1998) have proposed the presence of an unknown transcriptional activator termed ICE (inducer of *CBF* expression) that is present at warm temperatures. Upon exposure of a plant to low temperatures, ICE is proposed to be modified as a result of activation of the signaling cascade. ICE may then promote the increase or/and relieve inhibition of *CBF* gene expression, which leads to the cold acclimation response.

There is also growing evidence that calcium and phosphorylation play roles as early signaling components (Knight *et al.*, 1996, Monroy and Dhindsa, 1995, Sheen, 1996; Tahtiharju et al, 1997). Cold shock has been shown to cause an immediate increase in cytosolic free calcium that could be partially inhibited by EGTA or lanthanum (Knight *et al.*, 1996). By blocking calcium channels with lanthanum or EGTA, induction of cold-regulated gene expression was inhibited (Monroy and Dhindsa, 1995). Further supporting the role of calcium, at warm temperatures the addition of a calcium ionophore stimulated the influx of calcium and induced expression of some cold regulated genes (Monroy *et al.*, 1998). In addition to its possible role in cold shock, calcium release may be effecting a response from the *CBF* promoters as a result of circadian oscillations. Cycles of light or temperature are known to be entraining stimuli and calcium levels oscillate with circadian rhythm (McClung, 2001). Therefore, a transient spike in calcium levels after a light to dark transition may cause a transient activation of the *CBF* promoter similar to the increase in response after

a warm to cold shift. If the promoter is fused to a marker such as *GUS* which produces a stable protein, accumulated activity of the GUS protein would be visible after a few days of growth, which could explain the inability to use GUS staining to follow patterns of gene expression (Figure 2.7).

Calcium dependent phosphorylation/ dephosphorylation processes also seem to be involved in activating the cold acclimation process. The application of a protein phosphatase inhibitor stimulated the induction of the cold regulated gene *CAS15* while a protein kinase inhibitor blocked cold regulated gene expression (Monroy *et al.*, 1998). Jonak *et al.*, (1996) have also shown in alfalfa that a mitogen-activated protein kinase (MAP kinase) pathway is activated within 10 min of a plants exposure to low temperature.

Interesting ideas for upstream activation of *CBF* genes are suggested if one examines the effects associated with the application of the translation inhibitor cycloheximide . Mahadevan and Edwards (1991), Edwards and Mahadevan, (1992), and Cano *et al.*, (1994), while working on the induction of c-*jun* and c-*fos* found that cycloheximide could activate some protein kinases involved in signal transduction pathways even if concentrations of cycloheximide were not high enough to inhibit translation. Berberich and Kusano (1997) also found that subinhibitory concentrations of cycloheximide induce a calcium dependent protein kinase (*Zm*CDPK1) from maize. Thus a protein kinase, such as a MAPK or CDPK, may be activated in a signal transduction pathway in Arabidopsis by the addition of cycloheximide and substitute for low temperature or other stress requirements such as the release of calcium.

An additional level of regulatory control was suggested by a brief set of experiments described here involving treatment of plants with concentrations of cycloheximide believed to be high enough to prevent translation of *CBF* transcripts and subsequently prevent transcription of *COR* genes. We found in treated Arabidopsis plants, the level of *CBF* transcripts were elevated without the plants experiencing a cold treatment. The same concentration of cycloheximide did inhibit accumulation of *COR* genes after placing plants in the cold for several hours (HuanYing Qin, personal communication). One possible explanation for that is that some other factor that acts on the *CBF* promoter is a negative regulator and that rapid turnover and inhibition of new synthesis allows active transcription of the *CBF* genes at warm temperatures.

Currently the specific minimal *cis*-acting element sufficient to induce low temperature responsiveness to the *CBF* genes is not known. Additional rounds of promoter fragment fusion studies, site directed mutagenesis or electrophoretic mobility shift assays and DNase I footprinting would be helpful for locating such an element. Identification of a *cis*-acting element would aid in the isolation of binding factors such as the postulated ICE protein. However, the larger 155 bp fragment already known to be sufficient for low temperature responsiveness is possibly adequate to use for the capture of some binding factors. Such factors would then need to be further characterized to confirm their role in low temperature specific activation and to separate them from other stress responses if possible.

Materials and Methods

Bacteria and growth conditions. Cultures of *Escherichia coli* DH5α and *Agrobacterium tumefaciens* GV3101 were grown aerobically at 37°C or 30°C respectively, in LB broth (Sambrook *et al.*, 1989) or on solid LB agar medium containing 1.5% agar. Plasmid-containing cultures were maintained in a medium with selective antibiotics. Plasmids typically conferred resistance to kanamycin (Km, 50mg/L) or ampicillin (Ap, 100mg/L).

Bacterial DNA isolation and cloning. Plasmids were constructed using PCR (polymerase chain reaction) and standard molecular biological techniques (Sambrook, *et al.*, 1989). Primers used to amplify fragments of the *CBF* promoters contained sites for unique restriction enzymes so that after amplification, the PCR product could be restricted and then cloned directly into the final vector in one step.

Site directed mutations were created in regions of the specific promoter fragments by either Promega's Gene Editor (Madison, WI) or Stratagene's Quick Change (La Jolla, CA) mutagenesis kits according to the product guide. Promoter fragments and mutations were sequenced on an Applied Biosystems Automated Fluorescent Sequencer at the Michigan State University-DOE Plant Research Laboratory Sequencing Facility.

Plant transformation. The DNAs were transferred to *Arabidopsis* via a whole plant dipping method similar to that described by Clough and Bent (1998). Since the binary vector used to transfer the DNA to the plant also contained a bacterial gene which conferred resistance to Km, plants containing transferred

DNA were identified by germinating seeds on medium containing Km (50mg/L). These Km resistant seedlings were grown to maturity and the next generation seeds (T2) were often used for experiments.

Plant growth conditions. Plants were grown in soil and on agar medium. When grown in soil, seeds were scattered on the surface of pots of soil. The soil was kept moist and covered with clear plastic film to retain moisture until seeds germinated then the cover was removed. Seedlings were grown in chambers with 24h light (~120 μmol m^{-2} s^{-1}) at 22°C. Low temperature treatments were usually done in the same chamber using half of the light as when the plants were grown warm or they were placed in a refrigerated room at 4°C with dim light (~25 μmol m^{-2} s^{-1}). Humidity was not controlled.

Agar medium contained Gamborg's B5 nutrients (Life Technologies Inc., Gaithersburg, MD), and 1% phytagar (Life Technologies Inc., Gaithersburg, MD). Plate grown seedlings were kept at ~24°C with 16h light (~35-100 μmol m^{-2} s^{-1}) and 8h dark.

Treatments with ABA, cycloheximide or dehydration were performed by growing seedlings on filter papers that had been placed on top of agar so that the seedlings could be lifted off the plate with minimal damage or stress. For ABA and cycloheximide treatments, the filters with 10-14 day old grown seedlings were floated on solutions of ABA (100mM) or cycloheximide (10μg/ml) in covered dishes for various time periods. For dehydration treatments, filters with seedlings were removed from plates and placed in desiccation chambers for different time periods.

Mapping. Mapping was performed using the recombinant inbred lines of Lister and Dean (1993) derived from the ecotypes *Lansberg erecta* and Columbia (obtained from the *Arabidopsis* Biological Resource Center, Columbus, OH). DNA was extracted (Rogers and Bendich, 1988) from 99 recombinant inbred lines and used to map the position of the *CBF* gene and the four *COR* genes using the protocols described by Lister and Dean (1993) (obtained from the Nottingham *Arabidopsis* Stock Centre website http://nasc.nott.ac.uk/RI_data/how-to-man.html). Enzymes used to generate RFLPs for the genes *CBF2, COR6.6, COR15a, COR47* and *COR78* included *Dra*I, *Eco*RI, *Afl*II, *Eco*RV and *Dra*I, respectively. Segregation data was submitted to the Nottingham *Arabidopsis* Stock Center and analyzed using the MAPMAKER program (Lander *et al.*, 1987).

Staining for β-glucuronidase acitivty. In order to stain plant tissue to determine location of β-glucuronidase activity, experimentally treated plants were immersed in a GUS staining solution as described by Jefferson (1987) and incubated overnight at 37°C. Tissue was cleared with several rinses of 70% ethanol to aid in the localization of the staining. Images in this dissertation are presented in color.

RNA Isolation. Total RNA was extracted from plant material with the use of the Qiagen RNeasy Plant Mini Kits (Qiagen Inc., Valencia, CA) with modifications. To obtain adequate and consistent yields with the kit, the amount of starting plant tissue was doubled. Subsequently the amount of extraction

buffer (RLT) was also doubled. The remaining procedure was performed as described in the Qiagen manual.

RNA Blots. Prepared RNA samples were mixed with formaldehyde loading buffer containing ethidium bromide (~25ng/mL) and electrophoresed on formaldehyde agarose gels according to standard procedures (Sambrook *et al.*, 1989). RNA was transferred to nylon membrane in 20X SSPE according to standard protocols (Sambrook *et al.*, 1989). After transfer, the RNA was cross-linked to the membrane with UV light from a UV Stratalinker 2400 (Stratagene, La Jolla, CA). The membrane was prehybridized in a Robbins hybridization incubator model 400 (Robbins Scientific Corp., Sunnyvale, CA) in a solution containing 50% formamide, 5X SSPE, 5X Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS) and 100μg/ml sheared, denatured salmon sperm DNA. After 3-4 hours of prehybridization, ^{32}P-labeled probe was added to the solution to hybridize overnight at 42°C. The probe was prepared from a gel-isolated fragment by a random priming method (Feinberg *et al.*, 1983). After hybridization, the membrane was rinsed briefly in a solution of 2X SSPE and 0.5% SDS at room temperature followed by two washes at room temperature or 42°C for 10-30 minutes. The membrane was then washed 2-3 times for 15 minutes each at 50°C with a solution containing 0.1X SSPE and 0.5% SDS.

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Chapter 3

SCREEN FOR MUTATIONS IN THE SIGNAL TRANSDUCTION PATHWAY LEADING TO INDUCTION OF *COR* GENES

Summary

An experimental approach was designed for the identification of elements important in the low temperature signal transduction pathway leading to the induction of cold regulated (*COR*) genes. Several plasmids were constructed that contained a known cold responsive *cis*-acting element in the context of either the *COR15a* or *COR78* gene promoters from *Arabidopsis* and fused to several different marker genes. These plasmids were used to integrate the marker gene into plants. Plants were then chemically mutagenize then selected or screened for aberrantly regulated expression of the marker. Aberrant expression, either up-regulation of the marker under non-inducing conditions or loss of expression under inducing conditions, may indicate a mutation in the signal transduction pathway. Several plants were identified, which had diminished expression levels of at least one of the *COR* genes, and these mutant were further analyzed. RNA blot hybridization demonstrated however that in most cases when the transcript for a *COR* gene was absent or diminished, transcripts of the marker gene were also reduced. This indicated that some form of gene silencing was likely taking place and that the mutation was not in the signal transduction pathway. Means for improving the mutagenesis strategy to help identify signaling mutants are discussed.

Introduction

Because plants are generally sessile and cannot avoid environments that may be harmful to their survival, they have developed mechanisms to adapt to their environment as it continuously changes around them. Freezing temperature is one common adverse environmental factor that affects survival, productivity and plant distribution. Plants from temperate regions can generally be acclimated to freezing temperatures by first treating them with low nonfreezing temperatures. This cold acclimation process results from biochemical and physiological changes occurring in the plant. Some changes may include altered composition of membranes and proteins, changes in activity of some enzymes or accumulation of certain cryoprotective compounds such as sugars or polyamines. The expression of many genes is also up regulated in response to cold stress. One group of genes, the *COR* genes, consists of four stress responsive gene families, (*COR6.6, COR15, COR47*, and *COR78*), which are differentially regulated but with at least one gene in each family being induced by low temperature. These genes maintain many other common properties. The mRNA transcript levels increase within two hours of plants being transferred to cold (4°C) and remain elevated as long as the plants are maintained at low temperature. The transcript levels decrease rapidly when the plants are returned to normal growth temperature (Thomashow, 1994). The genes are also induced by dehydration stress or/and by the exogenous application of abscisic acid. Proteins encoded by the genes remain soluble upon boiling due to their high

hydrophilicity (Hajela *et al.*, 1990), which may be important during periods of stress that result in membrane-membrane interaction such as during freezing or dehydration stresses. One of the more thoroughly studied genes, *COR15a*, encodes a polypeptide that is targeted to the stromal compartment of the chloroplasts and can increase their freezing tolerance (Artus *et al.*, 1996). The increase in freezing tolerance appears to result from an interaction with the membrane that reduces the formation of deleterious hexagonal II phase lipids upon freezing-induced dehydration (Steponkus *et al.*, 1998).

The incentives for studying the mechanisms by which the *COR* genes are regulated include a desire to understand the basic strategies used in low temperature and other stress biology and also to use the information obtained to potentially bear against the cost of feeding an ever increasing population reliant upon a small group of crops forced to grow in environments that are less than ideal for producing their maximum yield. At the start of this work, a low-temperature and dehydration responsive *cis*-element had been identified in the promoter of the *COR* genes (Yamaguchi-Shinozaki and Shinozaki, 1993, Baker *et al.*, 1994) but no direct trans-acting factors nor any other upstream components had been identified that played a direct role in the their regulation.

Therefore, an attempt was made to identify some of the regulatory factors associated with the low temperature signal transduction pathway using several different cold responsive gene promoter fragments. Because there is no easily detectable phenotype specific for the induction of any of the *COR* genes, several marker genes were tested. The markers that were used included a herbicide

selection (*BAR*) which confers resistance to glufosinate ammonium, a negative selection marker (*codA*), which when induced can convert the innocuous 5-fluorocytosine (5-FC) into a toxic compound, and a phenotypic screen based on production of anthocyanin in mutant *Arabidopsis* plants that did not normally produce anthocyanin. A fourth marker gene encoding beta-glucuronidase (*GUS*) had been used in a similar study prior to this work (Wilhelm, 1996). The first three markers had potential benefits over *GUS* including use of either selective methods or the ability to nondestructively screen large populations of plants through isolation of phenotypic variation. All of these also had drawbacks that are discussed later.

A fifth marker system used by Ishitani *et al.* (1997) relied on the cold regulated *COR78* promoter fused to the luciferase gene. This screen has already identified many mutants affected in their response to low temperature and other stresses. The advantage of using luciferase is the ability to rapidly screen large populations of young seedlings with a high degree of sensitivity. Their success buoyed our hopes for finding some low temperature stress signaling genes.

It was hoped that new mutants could help identify components of the signal transduction pathway, especially if mutants altered in their response to a low temperature stimulus were also be altered in their response to other stresses. In addition, the number of mutants that could be isolated might have given a sense of the complexity of the signaling pathways involved. Several mutant lines isolated were identified with lower levels of transcript from at least

one *COR* gene in response to low temperature stress. These plants were further analyzed. In the end, however, no genes associated with low temperature signal transduction were isolated from any of the marker fusions and mutagenesis screens. Some information gleaned from experiments using these plants may benefit future experiments.

Results

Regulation of marker gene expression by low temperature. The choice of an appropriate promoter and marker gene is important for developing a screen for gene regulation mutants in the low temperature signal transduction pathway. Prior to this work a *COR15a* promoter fused to a *GUS* reporter was used to screen over 10,000 mutagenized plants without successfully identifying a mutant in the low temperature signal pathway (Wilhelm 1996). Subsequent to this screen, several promoter or promoter fragments and marker combinations were tested to identify the most useful. The first marker tested was for the bacterial gene *BAR* that encodes a protein which, when expressed in plants, confers resistance to herbicides with the active ingredient glufosinate ammonium (De Block *et al.*, 1987, Thompson *et al.*, 1987). Initially multiple copies or "multimers" of a *COR15a* promoter fragment containing the C-repeat (CRT) was fused to *BAR*. Earlier work with promoter/*GUS* fusions had shown that multimers of this CRT gave low temperature regulated expression in tobacco (Figure 3.1A). However, when this multimer was fused to *BAR* (or *GUS*) and transformed into *Arabidopsis*, expression was constitutive (Figure 3.1B). The multimeric form of the CRT was used because a smaller fragment of the *COR15a* promoter

containing only a single CRT did not result in any detectable transcript on an RNA blot (Figure 3.1B) or GUS protein as determined by colorimetric assays (Jefferson, 1987). A larger (approximately 1kb) fragment of the *COR15a* promoter was then fused to the *BAR* gene and also to a negative selection marker *codA* gene (Stougaard, 1993). The *codA* gene encodes a protein that can convert the normally innocuous compound 5-FC to the toxic compound 5-fluorouracil. These promoter-marker fusions were individually inserted into a binary vector that also contained the *COR15a* promoter fused to *GUS*

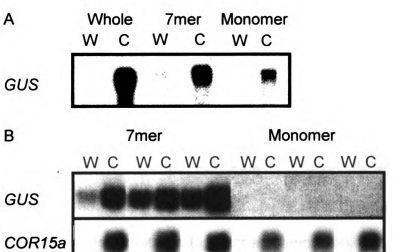

Figure 3.1. Expression pattern of *COR15a* promoter/*GUS* fusions. RNA blots demonstrate expression patterns of a *GUS* gene fused to a whole *COR15a* promoter, a multimer (7mer) CRT and a monomer CRT. Tobacco plants containing promoter fusions have cold induced transcript accumulation (A), while transgenic Arabidopsis plants high level of expression in warm and cold for the multimer and no transcript accumulation if the monomer is used (B). Each pair of samples from Arabidopsis represents an individual transgenic line. The endogenous *COR15a* gene transcript is also shown in B for comparison of expression at warm (W) and cold (C) treatment conditions.

resulting in a double marker vector. The constructs were then used to transform *Arabidopsis*. From the over 50 independent transformants generated for each construct, eight lines were chosen for further tests. These lines appeared normal when grown on agar medium and in soil and segregated 3:1 for kanamycin-resistant to kanamycin-sensitive in the T1 population suggesting a functional transgene insertion at a single locus for each line. For each line, plants homozygous for the transgene were identified and used to bulk seeds for later experiments.

Before mutagenizing plants, seedlings were grown under standard growth conditions to determine the suitability of the selection methods for isolation of mutants. To test the GUS marker, ten day old seedlings grown on solid agar medium were exposed to low temperature (4°C) for four days and stained according to Jefferson (1987). For all the lines, warm controls did not stain, and for all but one of the *BAR* containing lines when placed at low temperature, the plants stained blue indicating a functional *GUS* marker. These same lines were then grown and treated with either a solution containing glufosinate ammonium or 5-FC.

The plants containing the *BAR* gene marker were grown on either agar medium, sand or potting mix for 10-21 days before treatment with glufosinate ammonium. Glufosinate ammonium was then either sprayed on leaves of plants until run off or added to the growth medium. Concentrations ranging from 25μM to 2.5mM quickly killed non-transgenic control plants but in all cases some warm grown and cold treated transgenic plants containing the *BAR* gene survived.

Several different treatment regimes including changes in pretreatment growth conditions, age of treated plants and glufosinate ammonium concentrations never resulted in consistent killing of all warm grown transgenic plants.

The plants containing the *codA* gene were grown on solid agar medium with 5-FC either added to the medium or flooded onto the surface after plants were 10-14 days old. The 5-FC was added at either 1 mg/ml or 3 mg/ml. After growing in the warm, plates were transferred to a low temperature growth chamber. Several temperature regimes were tested since plants grew so slowly at 0-4°C that no effect of the 5-FC was observed. At temperatures above 12°C, the activity of the *COR15a* promoter is diminished and plants were less consistently affected by the 5-FC. A cycling of temperatures between 8-10°C for 16h per day and 22°C for 8h per day under continuous light was chosen because it was low enough to induce *COR15a* promoter driven expression of the marker and the warm period allowed faster growth. Greater than 90% of seedlings would eventually die after 2-4 weeks under this growth regime. However, seedlings had to be placed sparsely (<100 seedlings per 10 cm Petri plate) onto agar plates in order to achieve this level of selection. This would have required large amounts of space and considerable labor to screen through the number of seedlings expected to obtain mutants. And even at a 90% mortality level if the thousands of mutagenized seedlings had been analyzed, they would have generated too many false positives to make this selection method suitable for the large-scale testing required.

Finally a third marker, the *R* gene was fused to the *COR78* promoter (the plasmid was a gift from Mark Alfinito, Stanford University). The *R* gene encodes an anthocyanin specific transcriptional activator from maize, which is involved in the anthocyanin biosynthetic pathway and rescues a *transparent testa glabrous* (*ttg*) mutation in *Arabidopsis* (Lloyd *et al.*, 1992). The *ttg* mutant plants do not produce anthocyanin. However, when the *R* gene is introduced into the plants and driven by the *COR78* promoter (herein termed COR78pR plants), then plants start to produce red/purple colored anthocyanins in the leaves when grown at low temperature. Grown at warm temperature the plants appear light green in color.

Plants containing the *R* gene were grown for about 14 days in potting mix before initiation of cycling low temperature treatments of 10°C (16h) and 22°C (8h). The cycling temperature allowed faster growth and seemed to shorten the time for color to develop in leaves. Even so at least 12-14 days of this treatment was required for significant and even color development if a large population of seeds was planted in a small area.

From the numerous independent transformants, two lines were chosen for further experiments. One line contained a single transgene insertion and the other line contained two independent insertions as determined by DNA blot analysis (Figure 3.2). RNA was also isolated from these lines and their expression pattern from RNA blot analysis matched that of other *COR* genes (Figure 3.3 and Fig 3.4 parent lines).

Isolation of mutants with altered expression of the marker gene after low temperature treatments. About one gram of transgenic seeds from each

of the two COR78pR plant lines was mutagenized by EMS (ethyl methansulfonate) treatment essentially as described by Haughn *et al.* (1986), divided into 30 pools, germinated and the M1 plants were allowed to self-pollinate. The M2 seeds from each pool (about 0.5g each) were collected independently. M2 seeds from the different pools were scattered onto the surface of flats of potting mix and grown for about 10 days before starting low temperature treatments. During the screening it became evident that shading

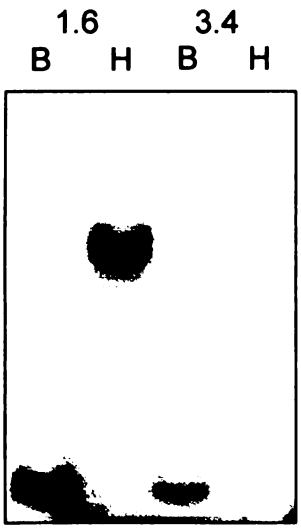

Figure 3.2. Determination of transgene copy number in transformed plants. DNA blot hybridization pattern observed for restricted genomic DNA isolated from the parent plant lines 1.6 and 3.4, which were subsequently used in mutagenesis experiments. The probe used for the hybridization was a ^{32}P labeled *R* gene fragment. Lanes labeled B contain DNA restricted with the endonuclease *Bgl* II, which cuts twice within the R gene but outside of the sequence used to make the probe. Lanes labeled H contain DNA restricted with the endonuclease *Hind* III, which cuts one time within the R gene but 5' to the fragment used to make the probe.

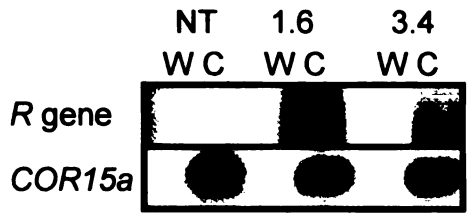

Figure 3.3. Gene expression pattern in transgenic plants. RNA blot hybridization demonstrating the normal expression pattern for the cold regulated *COR15a* gene and the cold regulated marker *R* gene. NT is a nontransgenic plant, 1.6 and 3.4 are transgenic plants containing the *R* gene regulated by the *COR78* promoter. W= warm grown plants, C= one day cold (4°C) treated plants.

from neighboring plants could cause a phenotype similar to what was expected for mutants therefore putative mutants lacking anthocyanin after a set length of time were transplanted out of the flats to individual cell pots and left in the treatment chamber an additional week. Those putative mutants that did not produce anthocyanin were transferred to normal 22°C growth chambers and allowed to set seed.

Approximately 150,000 M2 seedlings were screened and about 100 COR78pR plants were selected as putative mutants and grown for seed. This seed was then used to confirm the mutant phenotype. Of these, about 65 were confirmed mutants lacking anthocyanin when grown at low temperature and were used for further characterization by examining the expression of other *COR* genes and observing the effect on freezing tolerance.

Characterization of mutants. The effect of the mutation on expression levels of other *COR* genes was examined by RNA blot analysis. RNA was isolated from low temperature treated plants for each mutant and the parent non-mutagenized lines. The samples were electrophoresed, blotted and hybridized

with probes specific for the *COR* genes, (*COR 6.6*, *COR15a*, *COR47*, and *COR78*) as well as the *R* gene and a constitutively expressed *Arabidopsis eIF4A* (eukaryotic initiation factor 4A) gene (Metz *et al.*, 1992). The majority of mutants had no change in the level of expression for any gene except for the marker that was not expressed in several lines. A few had slightly decreased or complete loss of expression for *COR78* only but none had consistently lowered expression levels for all other *COR* gene (Figure 3.4).

Three of the most severe mutants with diminution of expression for *COR78* were tested for diminution of freezing tolerance as determined by the use of an ion leakage assay. Several independent ion leakage assays were performed and two assays are shown in figure 3.5. Each mutant sample and parent is represented by three replicates at each temperature. The variation in percentage of ion leakage between samples from a given temperature was large within a single assay and between the independent assays. This variability, inherent in the assay, may be due to handling differences between samples and non-uniformity in the tissues used for each sample. For example, no attempts were made to account for leaf age or size. Taking into account the sample errors, no significant change was observed in the temperature at which a 50% leakage point was reached between those plants that expressed the *COR78* gene normally and those in which the *COR78* gene expression had been reduced or eliminated. In both types of plants, 50% ion leakage had been surpassed at about -6°C.

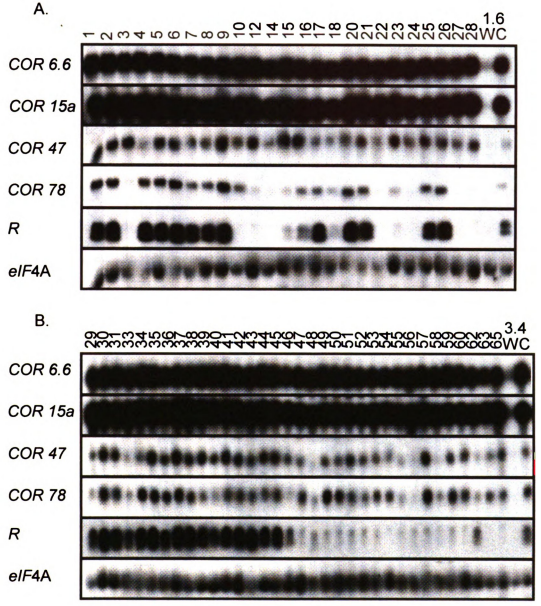

Figure 3.4. Gene expression in mutagenized plants. RNA blot hybridization expression pattern for *COR* genes, the *R* gene and *eIF4A* in different lines of plants that have been mutagenized. Parent lines 1.6 and 3.4 accumulate little transcript after growth at warm (W) temperatures, however abundant transcripts accumulate after cold (C) treatments. Putative mutants (1-65) selected by phenotypic loss of anthocyanin deposition in leaves show varying levels of transcript accumulation after cold treatment. Lines identified from the mutagenesis of parent 1.6 are shown in A. Lines identified from the mutagenesis of parent 3.4 are shown in B.

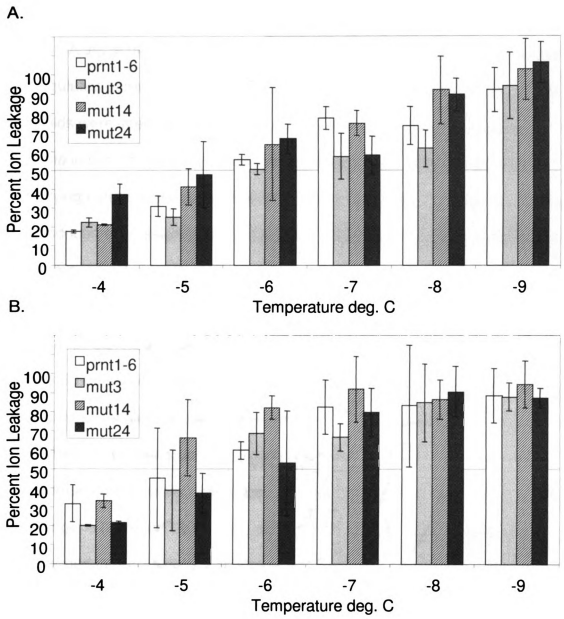

Figure 3.5. Ion leakage assays to determine cellular fitness or loss of fitness after a three day cold acclimation period. Two independent assays were performed (A and B) using three replicate tubes at each temperature. In both experiments, injury due to freezing resulted in cells losing 50% of their relative ion content at about 6°C for the control parent line, prnt1-6, and the three mutant lines.

Discussion

A set of plasmids was constructed containing the promoter from a cold up-regulated gene (either *COR15a* or *COR78*) fused to a marker gene. These plasmids were integrated into the genome of the model plant *Arabidopsis thaliana* in order to perform mutagenesis studies which could be used for identifying factors in the low temperature signal transduction pathway leading to cold induced expression of the known *COR* genes and other genes that share the same regulatory pathway.

Initially plasmids were constructed which contained multiple copies of the CRTs from *COR15a* fused to a marker. A single copy of the CRT still containing about 180 bases of context from the *COR15a* promoter had not given detectable expression in earlier tests and therefore the multimer was designed. A plasmid containing a multimer promoter sequence fused to a marker gene was initially transformed into tobacco, which resulted in cold up-regulated expression of the marker gene (Leonard Bloksberg, unpublished). When a similar plasmid was transformed into *Arabidopsis*, however, expression of the marker gene was elevated in both warm and cold to fairly high levels. The marker in *Arabidopsis* was the *BAR* gene which confers resistance to herbicides containing the active ingredient glufosinate ammonium. Several attempts were made to adjust the concentration of the glufosinate ammonium and change the growth medium and other environmental conditions including changing from agar medium to soil and increasing or decreasing light intensity and day length. Because at the time we could not explain the unexpected high level constitutive activity of the multimer

design in *Arabidopsis*, the decision was made to use a larger piece of the promoter which had been shown with the GUS marker to be dramatically up-regulated by cold (Wilhelm, 1996). Again, however, when the promoter was fused to the *BAR* gene, enough expression was observed in both warm and cold grown plants to confer resistance to the herbicide. Initially we were not able to account for the apparently constitutive low level expression of the promoter that resulted in resistance since at the level of detection by RNA blots, transcripts were generally absent in warm grown plants. In light of information obtained later while working with the *CBF* genes which are mechanically activated and possibly developmentally induced as well, we believe it is possible that input from additional stresses may have resulted in enough expression from the *BAR* gene to produce an active enzyme, which would be stable enough to disable the selectivity of the herbicide and prevent the use of the *BAR* gene as an effective tool. Alternatively, the glufosinate ammonium may have been directly stimulating expression of the *COR* genes and therefore the selection method would never be usable. This was not tested. These plants could not be used for a negative selection, that is, to identify genes in the cold signal pathway through loss of their activity, because loss of *BAR* gene activity would result in plants being killed when treated with the herbicide. Use of a later generational subset of seeds was not practical for the experiment. Also, the inconsistent killing of plants that had not been mutagenized indicated that there would have been a large number of false negative mutants to screen.

A second marker system based on the *codA* gene from the bacterium *E. coli* was then examined. Expression of this bacterial gene for cytosine deaminase should allow identification of candidate genes in the plant through a negative selection method. The method is based on the idea that since *Arabidopsis* does not normally contain the enzyme cytosine deaminase and does not metabolize cytosine to uracil, it should not be able to metabolize the innocuous compound 5-FC to the toxic compound 5-fluorouracil. While the selection method did not appear to have the problems with a constitutively active promoter since plants could be grown on selective medium containing the chemical 5-FC and survive in the warm, the killing in the cold was inconsistent. Here it appeared that mechanism for selection could work but not under the growth conditions needed to get adequate levels of expression from the *COR15a* promoter. Several attempts were made to modify the growth regimes and a minimally successful method was developed which relied in part, on cycling between warm and cold temperatures but the level of false negatives seen in non-mutagenized seedlings again would have been a hindrance to identification of plants containing real mutations in the cold signal transduction pathway. A more consistent method with the ability to screen through larger numbers of seedlings was needed. Such a method appeared to present itself in a system containing a group of *Arabidopsis* plants which completely lack anthocyanin and are devoid of trichomes (*ttg*, transparent testa glabrous mutants) and a marker gene *R* from the monocot maize which rescues the *Arabidopsis* mutation. When a cold inducible promoter regulates the *R* gene, anthocyanin is produced when

the plants are grown at low temperature. Because the method is a non-lethal screen, the *R* gene marker could possibly be used for both a positive and a negative screen. As a positive screen, plants would not produce anthocyanin in the cold if a mutation resulted in loss of expression of one of the genes in the low temperature signal transduction pathway. As a negative screen, plants would produce anthocyanin at warm growth temperature if a mutation resulted in increased expression of one of the pathway genes. Of course, one of the drawbacks to this screen is the potential for apparent loss or gain of *R* gene expression due to mutations not in the cold signal pathway but rather in the anthocyanin production pathway. Additionally, a single mutation in the promoter for the *R* gene may also cause a lack of anthocyanin production. The only method to identify such alternative mutants would be through RNA blot analysis of *COR* gene expression. Since the *R* gene is regulated by the same promoter as *COR78*, a decrease in expression affecting both genes is likely due to a mutation in the *COR* gene regulation pathway and not just in the promoter of the *R* gene or the anthocyanin pathway.

In the end the negative screen was not extensively utilized. However, by using the positive screen several plants were found that displayed loss of anthocyanin when grown in the cold and RNA blots showed a decrease in both the *R* gene and *COR78* gene expression. The expression levels of the other *COR* genes were not affected or only minimally affected. This result was not expected since we had predicted that the low temperature regulation of *COR* gene expression was taking place through the C-repeat which is present in all the

COR genes examined. This result suggested either a second pathway is being utilized for *COR78* regulation or possibly a form of gene silencing is taking place. Silencing caused by transcriptional inactivation may result from promoter homology between the endogenous *COR78* gene promoter and the introduced COR78pR transgene (Park *et al.*, 1996, Jakowitsch *et al.*, 1999). Neither of these hypotheses was tested.

Since no mutants were identified which affected all the *COR* genes, the plants with diminished *COR78* expression were analyzed alone for diminished tolerance to freezing. The ion leakage test is a rapid, albeit crude, measure for estimating freezing tolerance by examining the extent of cell damage resulting from freezing. Plant leaves are subjected to decreasing temperatures and later analyzed for damage relative to the amount of ions that have leaked from cells. While COR78 is but one of a large group of cold induced proteins its significance may have been estimated if mutants had shown a reduced capacity to cold acclimate. Unfortunately the difference between mutants and controls was not significant and so the relative importance of COR78 cannot be determined. Further characterization of these mutants was determined to be of minimal benefit due to lack of an effect on cold acclimation in mutants with reduced *COR78* expression, and the high possibility that the loss of expression was due to a silencing mechanism not associated with a mutation in the low temperature signal transduction pathway. Also due to the fact that Ishitani *et al.* (1997) had already identified several hundred potential mutants using a *COR78* promoter fused to the luciferase gene, this line of research was terminated. Their success

resulted mainly from their choice to use luciferase as a screenable marker because it allowed them to screen thousands of small seedlings on a single agar plate quickly and efficiently. Types of mutants they identified included those that lost the ability to activate expression of the *COR* genes after cold treatment as well as those that constitutively expressed *COR* genes at both warm and cold temperatures.

It is still possible that subtle differences in the regulation pathways of *COR78* and the other *COR* genes exist and there are a number of measures that could be taken to improve the chances of finding desired mutants in the signal transduction pathway. One would be to repeat an experiment similar to Ishitani et al., (1997) and use the luciferase gene as a screening marker. This marker would allow for rapid screens of large numbers of seedlings using several different stress treatments. T-DNA tagging to knock out genes and activation tagging methods to activate expression could be used in addition to EMS mutagenesis. A benefit of the tagging methods is the efficiency with which mutant genes can be identified, since sequences in the tag are known.

It is also possible that experiments could be developed using promoter fragments that are only inducible by low temperature to aid in the identification of components in the cold specific signal transduction pathway. The commonality of proposed functions for many of the cold, drought and salt tolerance genes makes it challenging to think which regulatory genes may or may not be specific to each stress tolerance pathway. Already Liu *et al.* (1998) have suggested a bifurcation in the pathways starting with *CBF/DREB1* and *DREB2*. Both are

transcriptional activators that bind to the C-repeat/dehydration responsive element but *DREB2* is specifically induced by drought and *CBF/DREB1* has been suggested to be up regulated specifically by low temperature (Liu *et al.*, 1998, Medina *et al.*, 1999). Although more recently the specificity of the regulatory mechanisms working on *CBF/DREB1* has been questioned. The *CBF/DREB1* genes do not appear to be solely responsive to low temperature and in fact respond to stresses like mechanical stimulation, ABA, drought and the chemical cycloheximide. Nevertheless, if studies using promoter fragments regulated by specific stresses could be developed, one might gain insights into how plants are able to distinguish between the different stresses and then use that information to more finely regulate responses of plants to changing environments.

Materials and Methods

Bacteria and growth conditions. Cultures of *Escherichia coli* DH5α and *Agrobacterium tumefaciens* GV3101 were grown aerobically at 37°C or 30°C respectively, in LB broth (Sambrook *et al.*, 1989) or on solid LB agar medium containing 1.5% agar. Plasmid containing cultures were maintained in a medium with selective antibiotics specific for the plasmids.

Bacterial DNA isolation and cloning. Standard molecular biological techniques were used for construction of all plasmids used in experiments (Sambrook *et al.*, 1989). Binary vectors containing the 7xCRT:*GUS* fusion and the *COR15a* promoter:*GUS* fusion were used in the basic constructs to which the 6xCRT:*BAR* and *COR15a* promoter:*BAR* or *COR15a* promoter:*codA* fusion fragments were added.

Plant transformation. The DNAs were transferred to *Arabidopsis* via a root transformation method (Valvekens *et al.*, 1988) or vacuum infiltration (Bechtold *et al.*, 1993).

Plant growth conditions. Plants were initially grown in Petri dishes on solid agar medium containing Gamborg's B5 (Life Technologies Inc., Gaithersburg, MD) and with kanamycin (50mg/L) to select for T1 lines containing the transgenes. Experimentally developed lines were grown under conditions, which would allow for selection with glufosinate ammonium (*BAR* gene containing plants) or 5-fluorocytosine (*codA* gene containing plants) or inducing conditions to screen plants by the *R* gene. Plants grown on plates were kept at about 24°C with 16h light (~35-85 μmol m^{-2} s^{-1}) and 8h dark. When grown in soil, seeds were scattered on the surface of pots of soil. The soil was kept moist and covered until seeds germinated then the cover was removed. Seedlings were grown in chambers with 24h light (~120 μmol m^{-2} s^{-1}) at 22°C. Low temperature treatments were usually done in the same chamber using half the intensity of the light as when the plants were grown warm or they were placed in a refrigerated room at 4°C with dim light (~25 μmol m^{-2} s^{-1}). Humidity was not controlled.

RNA Isolation. Total RNA was extracted from plant material using a lithium chloride precipitation procedure as described by Gilmour *et al.* (1988) or with the use of Qiagen RNeasy Plant Kits (Qiagen Inc., Valencia, CA) with modifications (Gilmour *et al.*, 1998). To obtain adequate and consistent yields with the kit, the amount of starting plant tissue was doubled. Subsequently the

amount of extraction buffer (RLT) was also doubled. The remaining procedure was performed as described in the Qiagen manual.

RNA Blots. Prepared RNA samples were mixed with formaldehyde loading buffer containing ethidium bromide (~25ng/mL) and electrophoresed on formaldehyde agarose gels according to standard procedures (Sambrook *et al.*, 1989). RNA was transferred to nylon membrane in 20X SSPE according to standard protocols (Sambrook *et al.*, 1989). After transfer, the RNA was cross-linked to the membrane with UV light from a UV Stratalinker 2400 (Stratagene, La Jolla, CA). The membrane was prehybridized in a Robbins hybridization incubator model 400 (Robbins Scientific Corp., Sunnyvale, CA) in a solution containing 50% formamide, 5X SSPE, 5X Denhardt's solution, 0.5% sodium dodecyl sulfate and 100μg/ml sheared, denatured DNA. After 3-4 hours of prehybridization, ^{32}P-labeled probe was added to the solution to hybridize overnight at 42°C. The probe was prepared from a gel-isolated fragment by a random priming method (Feinberg *et al.*, 1983). After hybridization, the membrane was rinsed briefly in a solution of 2X SSPE and 0.5% SDS at room temperature followed by two washes at room temperature or 42°C for 10-30 minutes. The membrane was then washed 2-3 times for 15 minutes each at 50°C.

***Arabidopsis* DNA Isolation and DNA blot analysis.** DNA was isolated from whole plants using a CTAB (hexadecyltrimethylammonium bromide) method (Doyle and Doyle, 1987). One gram of fresh tissue was ground in a mortar with a pestle after freezing in liquid nitrogen and placed in a plastic tube to which 3mL

of a 2X CTAB buffer (2% CTAB, 1.4M NaCl, 0.02MEDTA, 0.1M Tris pH8) were added. The tube was incubated for 30 min at 60°C. After cooling, the sample was extracted 2-3 times with chloroform then the DNA was precipitated with 2M NaCl and 0.75 volumes isopropanol. After pelleting the DNA by centrifugation, the DNA was suspended in 0.01M Tris/0.001M EDTA and precipitated again with 1/10 volume of 3M sodium acetate and 1 volume 95% ethanol. After centrifugation the pellet of DNA was suspended in water.

Total DNA was digested with various restriction enzymes and the fragments separated by agarose gel electrophoresis. The DNA was then transferred to nylon membranes according to standard protocols (Sambrook *et al.*, 1989). The DNA was UV cross-linked to the membrane and hybridized in a manner identical to that described for RNA blot hybridization.

Ion leakage. The percent of ions leaked from plant leaf tissue at various temperatures was determined using an ion leakage test described by Jaglo-Ottosen, *et al.*, (1998).

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Chapter 4

PERSPECTIVES AND FUTURE DIRECTIONS

Introduction

Average crop yields in the United States are three to seven times lower than that of record yields and this large discrepancy is thought to be caused by unfavorable environmental conditions affecting plant growth (Boyer, 1982). A significant environmental factor contributing to the loss is low temperature stress. This is part of the reason why the study of low temperature stresses has become such an important area of research. In addition, our expectations for plants productivity and growth often extend beyond evolutions initial design. Many of the major crops grown in the temperate zones of the world such as maize, rice, some of the bean crops, tomatoes, and cucurbits evolved in semi-tropical regions of the world where development or maintenance of tolerance to freezing temperature was not necessary. Accordingly, many of these plants are susceptible to low temperature stresses.

The early spring plantings and late fall harvests required by the extended growing season of many crops lead to unfavorable growth environments and yield loss. Thus the goal of many plant breeders has been to increase the tolerance of crops to all manner of environmental stresses including freezing stress. Traditional breeding has had limited success however and there has been little improvement in the freezing tolerance of many important crop species over the last several decades (Thomashow 1990; Sarhan *et al.*, 1998).

Frequently wild relatives of cultivated varieties of plants contain the genetic information to increase freezing tolerance. However these same wild relatives often also contain traits that are undesirable which adds to the difficulty of traditional breeding for improved stress tolerance. And because freezing tolerance is a quantitative trait, expression of several genes need to be integrated and regulated in any crop plant that is being modified and this is not easily done using traditional breeding technology. With advances in biotechnology and genomic information available from several different crop plants and the model plant *Arabidopsis*, the use of molecular biological techniques may now afford researchers the ability to selectively manipulate specific aspects of physiological/biochemical pathways to increase a plants cold tolerance. In order to do this, one still needs to understand the mechanisms of regulation and identity of factors that are associated with low temperature responsive gene expression.

Future directions

Many cold-responsive genes are likely to be found over the next few years. With the use of gene chip technology and full genome Arabidopsis (and other plant) arrays, genes involved in signal transduction pathways may begin to appear more frequently in these lists. Transcription factors such as the *CBF*s have proven themselves to be valuable tools for increasing stress tolerance (Jaglo-Ottosen et al., 1998), however high levels of these factors constitutively expressed can have negative impact on plant productivity as well (Gilmour et al., 2000).

In the present study, a 155bp fragment of the *CBF2* promoter was found to be sufficient to confer low temperature regulated expression to a marker gene. This region was also responsive to mechanical agitation, ABA and cycloheximide. Mutations created in several locations were unable to completely eliminate the regulated response to stresses. This led to the conclusion that more than one *cis*-element may be present and that multiple *trans*-acting factors including an inducer and possibly an inhibitor of *CBF* expression may be at work. Attempts to use computer software to scan the promoters of the three known *CBF* promoters for likely *cis*-elements has had limited success to date. One of the next steps may include using gel shift and competition assays to narrow the region in the 155bp fragment that binds a specific protein. Foot printing experiments may also be useful in this regard. If the region(s) of binding can be narrowed, mutations can be made in the locations and transgenic plants can be generated to test the *in vivo* effects of the loss of an element. This work would still only give a *cis*-element but it would greatly enhance the ability to use technologies like the yeast one-hybrid and southwesterns to identify *trans*-acting factors. These techniques to date have been unsuccessful largely due to high background from nonspecific binding (Sarah Fowler and Sarah Gilmour, unpublished).

An alternative mutagenesis approach to identify *trans*-acting factors may still benefit the research. Similar approaches using *GUS* as a marker were unsuccessful because of problems with high protein stability. However, the use of luciferase as a marker may be a key. Initial work with luciferase was similar to

GUS with high background at warm temperatures but new techniques in which the background activity is first eliminated by the addition of high concentrations of substrate (luciferin) have been developed. Only new luciferase production is then quantified. A *CBF2* promoter/luciferase construct is being tested in plants and may hold some promise (Daniel Cook, unpublished).

A similar mutagenesis approach could be developed using a *COR* gene promoter fused to luciferase. Ishitani et al., (1997) used the *COR78* promoter fused to luciferase and found several classes of mutants affecting *COR* gene expression. A *COR15* promoter fusion would likely find similar classes of genes. These mutants could also be screened by RNA blot hybridization to determine the specific effects on *CBF* expression. Some of these mutants would presumably contain defects in the signal transduction pathway and would be interesting to study.

Mutants that do not affect *CBF* yet still inhibit *COR* gene expression would also be interesting to study. Knight et al., (1999) have shown that one mutant, *sfr6*, suppresses the low temperature induced *COR* genes but does not effect *CBF* expression. The function of *sfr6* is not known but it may play a role in either activating CBF proteins at low temperature or assisting with binding specificity. Other proteins with similar functions may be identified with a mutagenesis screen.

Conclusions

Finally, many issues concerning the *CBF* genes have been presented since their initial discovery and many remain mysteries. For example, why is

there such redundancy, are there specific tissues in which individual *CBF* genes are more likely to respond or are there specific conditions like cold versus drought when one of the *CBF* genes is more responsive? Three copies of the *CBF* genes are located in direct repeats in the Arabidopsis genome (Gilmour et al., 1998) and more recently an additional three *CBF*-like genes have been found elsewhere. At present little is known about the function of the other *CBF*-like genes. An additional class of CRT/DRE binding factors, the *DREB2*s, are involved in increasing drought and ABA responsive expression of CRT/DRE containing genes (Liu et al, 1998). These redundancies may just be due to the importance of the need to cope with multiple stresses in a natural environmental condition with a rapid and strong response. There may also be subtle differences in types of stress or tissues in which individual genes function and we have just not yet teased apart the differences.

Of the many cold responsive genes known in Arabidopsis and other plants, their role in acclimating plants to low temperature is known for only a few. This leaves the challenge of trying to assign roles for individual gene products and determining their significance in the cold acclimation response. Very few regulatory/signaling components are known and this is an important group. Genes like *CBF* whose products govern the activation of multiple components of the cold acclimation response pathway can have profound effects on freezing tolerance. The discovery of more regulatory genes in other non-*CBF* pathways may have additional importance for designing schemes to help crop plants cope with environments less favorable for growth and productivity.

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