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ABSTRACT

ROLE OF THE ARABIDOPSIS *CBF* FAMILY OF TRANSCRIPTION FACTORS IN PLANT COLD ACCLIMATION

By

Kirsten Ruth Jaglo

Many plants, including Arabidopsis and canola, increase in freezing tolerance after exposure to low, nonfreezing temperatures, a process called cold acclimation. Numerous physiological and biochemical changes are associated with cold acclimation including changes in gene expression. The *COR* (cold-regulated) genes are associated with cold acclimation, and their expression is greatly increased under acclimating conditions due to multiple copies of a cis-acting DNA regulatory element called the CRT/DRE. A transcription factor called *CBF1* (CRT/DRE-binding factor one) was isolated which can bind to the CRT/DRE sequence and activate transcription in yeast. Overexpression of *CBF1* in Arabidopsis resulted in increased *COR* gene expression and increased freezing tolerance without a low temperature stimulus. Further research has shown that *CBF1* is a member of a small gene family that also includes *CBF2* and *CBF3*. RNA accumulation data indicated that all three *CBF* genes are activated under acclimating conditions. However, despite repeated attempts using both immunoblot analysis and immunoprecipitation, CBF protein accumulation could not be detected in wild type or *CBF*-overexpressing plants. Sequence analysis indicated that a putative *CBF* homologue, *BnCBF*, exists in *Brassica napus* (canola), a close relative of Arabidopsis. A time course of *BnCBF* RNA accumulation showed a similar induction pattern to that seen

with the Arabidopsis *CBF* genes. Overexpression of the Arabidopsis *CBF* genes in *B. napus* var. Westar, a spring variety of canola, showed increased *BN* gene (*COR* gene homologue) RNA accumulation and increased freezing tolerance under both nonacclimating and acclimating conditions. Additionally, increases in total soluble sugars were seen under nonacclimating and acclimating conditions, and increases in free proline under were seen under acclimating conditions. In summary, the *CBF* family of transcription factors appears to play important roles during cold acclimation in Arabidopsis and its close relative, canola.

To my grandpa:

William George Jaglo

who understood the language of flowers,
and
never
stopped learning.

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"What lies behind us and what lies before us are tiny matters compared to what lies within us"

Ralph Waldo Emerson.

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PREFACE

In Chapter 2, all experiments were conducted by the author of this thesis, except for the RNA accumulation analyses in Figure 2.2A which were conducted by Daniel Zarka, and the immunoblot analysis in Figure 2.2B which was conducted by Sarah Gilmour. Most of the results from this chapter were published in *Science*. 280: 104-106.

In Chapter 3, all experiments, including immunoblot analysis and immunoprecipitation experiments were conducted by the author of this thesis. The constructs encoding for the various portions of GST-labeled CBF1 were made by Eric Stockinger. For use in immunoblot analysis experiments, protein extracts from *CBF1*-overexpressing and vector control yeast were kindly donated by Eric Stockinger. For use in immunoblot analysis and immunoprecipitation experiments, proteins isolated from Arabidopsis nuclei were kindly donated by Charlie Herman, and proteins isolated from Arabidopsis protoplasts were kindly donated by Yaopan Mao. The transgenic *CBF*-overexpressing Arabidopsis lines, G7a-1, E71-1, A30a-1, A38b-7 and the vector control line B16-1 were made by Maite Salazar and Audrey Sebolt.

In Chapter 4, all transgenic canola plants used in experiments were generated by Susanne Kleff. All experiments, including RNA accumulation, immunoblot analysis, proline analysis, total soluble sugar analysis and electrolyte leakage analysis were conducted by the author of this thesis. In the salt stress experiments, the first experiment was conducted by Susanne Kleff, the second experiment was conducted in collaboration with Susanne Kleff and the third experiment was conducted by the author of this thesis.

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1. CHAPTER 1: Effects of Chilling and Freezing Temperatures on Plants and Changes Associated with Cold Acclimation

1.1 INTRODUCTION

Environmental stress consistently causes a decrease in maximum crop yield worldwide. It is estimated that 60% of potential yield is lost annually due to adverse environmental conditions (Levitt, 1980). One category of adverse environmental conditions is low and freezing temperatures. Depending on the region of origin, different species of plants show differing abilities to withstand chilling and freezing temperatures. For example, plants originating from tropical and sub-tropical climates that have not evolved the ability to withstand low temperatures, become damaged and may die from chilling temperatures of 10°C or less (Kratsch and Wise, 2000). In contrast, plants from temperate regions have evolved the ability to withstand low and even sub-zero temperatures by cold acclimating, a process by which plants increase in freezing tolerance after being exposed to low, non-freezing temperatures (Thomashow, 1999). This difference in the ability to survive low and freezing temperatures has a major impact on where crops are planted, when crops are planted, and the specific type of crop that is planted in a given geographical location.

At present, enough food is produced to feed the world (although not everyone is adequately fed due to complex problems with distribution) (Engelman and LeRoy, 2000).

However, there is reason to be concerned that there may not be enough food in the near future. The world population, currently at 6 billion, is predicted to reach 8.9 billion by 2050 (Engelman and LeRoy, 2000). Additionally, the amount of arable land per person is expected to decrease from 0.44 hectares to 0.16 - 0.18 hectares by 2025 (Engelman and LeRoy, 2000). To produce enough food, there will need to be an overall increase in crop yield and/or an expansion of agricultural production areas. One way to increase both is to improve the ability of plants to withstand freezing temperatures, both before and after cold acclimation. This would increase yield by reducing cold-induced damage, such as that experienced after a sudden frost, and by allowing higher yielding varieties to be planted in areas that are currently unsuitable due to inhibitingly low temperatures. For example, winter varieties of canola (*Brassica napus* and *Brassica rapa*) have 40% higher yield than spring varieties, making them more desirable to grow. However, only spring varieties are planted in some geographic locations as winter varieties cannot survive the extreme low temperatures experienced during the winter months (http: //www.canola-council.org/). Agricultural expansion could also occur as more freezing tolerant plants could be sown in areas that are not currently suitable due to temperature restraints.

Although improving freezing tolerance has been a long-term goal of plant breeders, the most cold tolerant varieties today are only marginally better than those produced at the turn of the century (Thomashow, 1990). Increasing our understanding of cold acclimation by focusing on the molecular changes that occur during acclimation may lead to better strategies in improving freezing tolerance.

1.2 EFFECTS OF CHILLING TEMPERATURES ON PLANTS

Damage due to low temperatures can be separated into two general categories, chilling damage and freezing damage. Chilling damage occurs in plants from tropical and sub-tropical regions, at temperatures below ~10° C (Kratsch and Wise, 2000). The extent to which plants are damaged is highly species specific and depends on the level of irradiance, the level to which the plant is hydrated at the time of the stress and the duration of the temperature stress. Despite these variables, there are general trends observed in chilling sensitive species. The chloroplast is the first organelle in which ultrastructural chilling damage can be observed. Damage includes swelling of the thylakoids, reduction in size and number of starch granules, unstacking of the grana, and depending on the duration of the stress, the eventual disintegration of the chloroplast (Kratsch and Wise, 2000). As chloroplasts are essential to plant life, even moderate damage can be lethal.

The molecular basis behind the chilling-induced damage to chloroplasts is complex. However, the ultimate result is the inhibition of photosynthetic carbon fixation (Strand et al, 1999), which can result in the death of the plant. In contrast to chilling tolerant plants, chilling sensitive plants do not recover their photosynthetic capacities after a chilling stress (Klimov et al, 1997). At low temperatures, chilling sensitive species show a reduction in photosynthetic gene expression. This inhibits the ability of a plant to harvest the light energy entering the chloroplasts and causes production of free oxygen radicals that damage the plant. Changes in membrane fluidity during low temperature stress also result in increased damage to chloroplasts (Strand et al, 1999) as the efficiency

of electron transport in photosystem II is reduced, resulting in the increased production of damaging free oxygen radicals.

1.3 EFFECTS OF FREEZING TEMPERATURES ON PLANTS

Freezing stress can result in protein denaturation, the production of free radicals, and damage to cellular membranes. A primary site of freezing damage in plants is the cellular membrane system (Steponkus, 1984). As the temperature drops below 0° C, ice formation occurs in the intercellular spaces as these contain fewer solutes than intracellular fluid and therefore have a higher freezing point. The chemical potential of ice is less than that of liquid water, so the intracellular osmotically active water moves down the water potential gradient toward the lower potential in the intercellular spaces and leaves the cell until chemical equilibrium is reached (Thomashow, 1999). Plants frozen at –10°C generally lose more than 90% of their osmotically active water (Steponkus and Lynch, 1989).

The damage that occurs to membranes during freezing is due to freeze-induced dehydration, which mimics damage induced by dehydration and osmotic stress (Steponkus, 1984; Steponkus et al, 1993b). The primary manifestation of damage caused by freeze-induced dehydration stress is dependent on the absolute temperature reached, but includes expansion-induced lysis, lamellar-to hexagonal-II phase transitions and fracture jump lesions (Steponkus et al, 1993a; Uemura and Steponkus, 1997).

The most common type of freeze-induced damage that occurs in nonacclimated plants is the formation of hexagonal-II phase transitions. These occur when the plasma

membrane is brought into close apposition to intracellular organelle membranes during freeze-induced dehydration. As the membranes are in close proximity to each other and water and other polar molecules are in limited supply, it is no longer energetically favorable for the nonpolar fatty acid portion of the membrane to remain in a lipid bilayer. The lipids can become reoriented and combine with lipids in other membranes into long cylinders with their polar head groups in an aqueous pore (Steponkus, 1984; Uemura et al, 1995). When water returns to the cell after the plant is returned to non-freezing temperatures, the lipid membranes do not reform into independent lipid bilayers and depending on the amount of membrane rearrangement that has occurred, severe damage can occur.

Expansion induced lysis can also occur with return of water to the cell. (Steponkus, 1984). This occurs as endocytotic vesicles bud off from the plasma membrane during freeze-induced dehydration as the cells shrink in size. After thawing, the vesicles are not reincorporated into the membrane resulting in the inability of the cells to accommodate the influx of water (Steponkus and Lynch, 1984).

Free oxygen radicals may also be produced during freezing stress. The radicals are the result of the reduced ability of chloroplasts to harvest the light energy entering the cell, due, in part to changes in membrane fluidity during freezing temperatures (McKersie and Bowley, 1997; Smirnoff, 1998). If many free oxygen radicals are produced, the plant can experience high levels of damage. Additional damage may result from protein denaturation or aggregation due to temperature induced conformational changes (Smirnoff, 1998). Depending on the protein affected, an important enzymatic function may be lost or changed and damage may ensue.

1.4 COLD ACCLIMATION

1.4.1 INTRODUCTION

Cold acclimation is the process by which plants increase in freezing tolerance after exposure to low non-freezing temperatures. Cold acclimation involves the activation of a series of physiological and biochemical changes that reduce the amount of damage to plants caused by both chilling and freezing temperatures. It is associated with changes in sugar accumulation, lipid composition, isozyme patterns, total protein content (Weiser, 1970) and changes in protein phosphorylation (Monroy et al, 1993). It is a multi-genic trait that allows freezing-tolerant plants to survive temperatures that are lethal to the same plant in the nonacclimated state. However, exactly which of these changes are the result of a metabolic response to the decrease in temperature, which are induced as a function of cold acclimation, and how such changes are activated has remained unclear. In the following sections, I will focus on some of the key changes associated with cold acclimation that relate to this thesis.

1.4.2 INVOLVEMENT OF ABSCISIC ACID (ABA)

ABA has long been considered a stress hormone. When plants are exposed to cold or other types of osmotic stress conditions, there is a burst of ABA production (Thomashow, 1999). Exogenous application of ABA to unstressed plants can result in

biochemical changes similar to those seen under conditions of cold/osmotic stress (Hare et al, 1999). If nonstressed plants are first treated with ABA and then exposed to osmotic stress, such as freezing temperatures, drought or salt stress, they frequently have increased tolerance to these stresses as compared to untreated nonstressed plants (Hare et al, 1999). These data suggest that ABA production and signaling are involved in cold acclimation as well as in acclimation to other osmotic stresses. However, even under conditions of continual stress, ABA levels eventually decline to those seen in unstressed plants (Lang and Palva, 1992), whereas freezing tolerance continues to increase for up to a week (Gilmour et al, 1988).

To investigate the role of ABA in cold acclimation, experiments were conducted in plants with mutations in either ABA sensing (*abi1*) or in ABA production (*aba1*) (Koornneef et al, 1998). While plants with either mutation are more sensitive to freezing than wild type plants, both types of plants also have compromised health overall, making it difficult to determine if the reduction in freezing tolerance is a primary or secondary effect of aberrant ABA signaling (Gilmour and Thomashow, 1991). Another way to determine if ABA plays a role in cold acclimation is to investigate induction patterns of known cold induced genes in the two mutant backgrounds. If cold induced genes have typical induction patterns under acclimating conditions in the mutant background, then ABA is clearly not necessary for cold-induced activation of these genes. In fact, this appears to be the case. The cold induced activation of the *COR* (cold-regulated) genes (discussed in detail in section 1.4.6.2) was not impaired in *abi1* or *aba1* plants, whereas the ABA-induced activation of these genes was lost (Gilmour and Thomashow, 1991). These data clearly indicate that there are two possible induction pathways of the *COR*-

genes, an ABA-dependent and an ABA-independent pathway. Investigation of other genes involved in stress induced pathways, such as those involved with the accumulation of free proline, also show both ABA-dependent and ABA-independent induction patterns (Hare et al, 1999).

1.4.3 Effects on Chloroplasts

When plants are first shifted to low temperatures, inhibition of photosynthetic activity occurs in chloroplasts which can result in free oxygen radical production and damage to the plant (Klimov et al, 1997). Therefore, some of the changes associated with cold acclimation function to protect chloroplasts and prevent permanent inhibition of photosynthetic carbon fixation (Klimov et al, 1997; Strand et al, 1997; Strand et al, 1999). For example, under acclimating conditions, chloroplasts increase in size (Klimov et al, 1997) and changes occur in the lipid composition of the inner and outer chloroplast envelope which reduce the occurrence of hexagonal-II formation and fracture-jump lesions in rye leaves (Uemura and Steponkus, 1997). To preserve photosynthesis, an increase in activity in seven of the enzymes associated with the Calvin cycle occurs (Strand et al. 1999). When Arabidopsis plants are grown under the acclimating condition of 5° C, a five-fold increase in carbon fixation rates occurs and large pools of soluble sugars are present in the chloroplast without suppression of photosynthetic gene expression or metabolism (Strand et al, 1997; Strand et al. 1999).

Cold acclimation also appears to reduce damage to chloroplasts by limiting the production of free oxygen radicals. In potato, superoxide dismutase isoenzymes are

activated under acclimating conditions, presumably to reduce free oxygen radicals created by the initial inhibition of photosynthesis (Seppanen and Fagerstedt, 2000). Free radicals are also produced during freezing in winter wheat and cold acclimated plants are more resistant to the addition of free radicals than nonacclimated plants (Kendall et al, 1989). The importance of this reduction is also seen by the observation that winter survival in alfalfa plants overexpressing iron superoxide dismutase increases compared to control plants (McKersie et al, 2000).

1.4.4 CHANGES IN MEMBRANES

Cold acclimation is associated with overall changes in the lipid composition of cell membranes that result in increased cryostability (Uemura et al, 1995; Uemura and Steponkus, 1997). The exact changes that occur are species and organ specific (Uemura et al, 1995), as is the absolute amount of the increase in freezing tolerance. In the case of the plasma membrane, the changes result in the production of exocytotic extrusions under freeze-induced dehydration as opposed to the more damaging endocyotic vesicles that are produced if nonacclimated plants are frozen (see 1.3, Steponkus et al, 1988; Uemura et al, 1995). In chloroplast membranes, the lipid changes result in modifications that reduce their propensity to form freeze-induced hexagonal-II phase transitions (Uemura and Steponkus, 1997). As the plasma and chloroplast membranes are considered the most vulnerable to freeze-induced dehydration stress, increasing the cryostability of these membrane systems is critical to increasing overall plant freezing tolerance (Uemura et al, 1995; Uemura and Steponkus, 1997).

1.4.5 CHANGES IN SMALL MOLECULES

Upon exposure to low temperatures, drought, or other forms of osmotic stress, plants accumulate a number of small benign molecules known as compatible solutes (Smirnoff, 1998). Examples of the solutes produced include sugars, such as sucrose and glucose, sugar alcohols, low-complexity carbohydrates, sulfonium compounds, and amino acids, such as proline (Bohnert and Sheveleva, 1998). The functions of these molecules is predicted to be the maintenance of turgor by lowering the osmotic potential, protecting macromolecules from denaturation, and protecting and stabilizing membranes (Smirnoff, 1998; Steponkus, 1984). It has been postulated that the increased accumulation of sugars and proline could be the result of disturbances in metabolism that occur under acclimating conditions (Bohnert and Sheveleva, 1998). However, while increased levels may in part be due to metabolic imbalance, there is also good evidence to suggest that accumulation increases through cold/osmotic-induced activation of the sugar and proline production pathways (Hare et al, 1999). Overexpression of a cold-induced transcription factor called *CBF3* (see 1.4.6.6) results in the increased accumulation of sucrose and other soluble sugars and increased activation of P5CS, which increases proline production (Gilmour et al, 2000).

Sugars are thought to function as cryoprotectants and have been shown to protect membranes against freeze-induced damage *in vitro* (Strauss and Hauser, 1986; Anchordoguy et al, 1987). Proline also appears to be able to increase freezing tolerance as seen by the work of Kobayashi and colleagues (1999). By creating Arabidopsis plants

that overexpress the proline dehydrogenase gene (ProDH) in the antisense orientation, transgenic plants with increased proline accumulation were isolated. Compared to control lines, the transgenic plants showed increased tolerance to freezing and salinity stress, indicating that proline accumulation is positively correlated with stress tolerance (Nanjo T, et al, 1999). While data on sugars and proline clearly indicate that both play roles in increasing freezing tolerance, the exact mechanism by which both function to produce this increase remains to be determined.

1.4.6 CHANGES IN GENE EXPRESSION

1.4.6.1 Introduction

In 1970, Weiser (1970) proposed that cold acclimation involved changes in gene expression. This was first conclusively demonstrated in 1985 in spinach (Guy et al, 1985) and extended to Arabidopsis in 1988 (Gilmour et al, 1988). Since these discoveries, many other groups have also found and characterized changes in gene expression in numerous other species such as canola, (Orr et al, 1992; Weretilnyk et al, 1993) cabbage, (Sieg et al, 1996), wheat, (Houde et al, 1992), barley (Crosatti et al, 1996; Phillips et al., 1997) and alfalfa (Wolfraim et al., 1993; Monroy et al., 1993). Some of the changes in gene expression involve proteins of known activities. One such set of proteins is the antifreeze proteins, which inhibit ice crystal growth and block small crystals from recrystalizing into larger crystals (Griffith et al, 1992; Yu and Griffith, 1999). Other proteins include lipid transferases, alcohol dehydrogenases, translation elongation factors (Nishida and

Murata, 1996), phenylalanine ammonia-lyase and chalcone synthase (Levya et al, 1995) to name a few. Still other changes involve proteins of unknown functions.

1.4.6.2 COR *genes*

In Arabidopsis, some of the best characterized cold-inducible genes are the *COR* (cold-regulated) genes also called - *LTI* (low temperature-induced), *KIN* (cold-inducible), *RD* (responsive to desiccation) and *ERD* (early dehydration-inducible), which are also induced by drought stress and the application of ABA (Nordin et al, 1991; Wang et al, 1994; Welin et al, 1994). The Arabidopsis *COR* genes described to date are *COR6.6, COR15, COR47* and *COR78*, all of which have homologues located in tandem in the genome (Thomashow et al, 1997). The *COR* genes encode proteins that are all highly hydrophilic, soluble upon boiling in aqueous buffer, and are predicted to form amphipathic α-helices (Lin and Thomashow, 1992; Thomashow, 1993; Thomashow, 1999). The COR78, COR15 and COR6.6 polypeptides are novel while COR47 has similarities to LEA type II proteins, also known as dehydrins, which are associated with desiccation and drought (Dure et al, 1989; Gilmour et al, 1991; Thomashow et al, 1993; Close, 1997).

COR gene homologues are present in diverse freezing tolerant plant species. In *B. napus*, *BN28* (Orr et al, 1992) is a homologue of Arabidopsis COR6.6 and *BN115* (Weretilnyk et al, 1993) is a homologue of Arabidopsis COR15, in wheat, *wcs120* (Houde et al, 1992), in barley, *HVA1* (Heino et al, 1990; Hong et al, 1992) and in alfalfa, *cas15* (Monroy et al, 1993). These data suggest that *COR* gene homologues exist

throughout angiosperms and may play important roles in increasing the freezing/osmotic stress tolerance of plants.

1.4.6.3 *Overexpression of* COR15a

The *COR* genes are clearly associated with cold and drought stress. This therefore raised the question as to whether they actually play roles in increasing freezing tolerance. To answer this question, Arabidopsis plants that constitutively overexpress *COR15a* were created (Artus et al, 1996). Previous experiments had shown that COR15a is imported into the stroma of the chloroplast and processed into a mature protein designated COR15am (Artus et al, 1996; Lin and Thomashow, 1992; S. Gilmour and M. Thomashow, unpublished). Artus and colleagues determined that *COR15a*-overexpressing plants have a 1-2° C increase in the freezing tolerance of chloroplasts and isolated leaf protoplasts between the temperature range of –4 and –8°C as compared to wild type plants. However, when investigating the freezing tolerance of detached leaves by electrolyte leakage analysis, or by conducting whole plant freeze tests, there were no differences seen between *COR15a*-overexpressing plants and wild type plants (Artus et al, 1996; Jaglo-Ottosen et al, 1998). Fluorescein diacetate staining of the protoplasts, an indicator of whether or not plasma membranes have retained their semi-permeable nature, indicated that COR15a overexpression protected the plasma membrane between –5 to –8°C (Artus et al, 1996).

1.4.6.4 Function of COR15a polypeptide

Since overexpression of the COR15a protein results in increased freezing tolerance, how the protein functions to bring about this increase became of interest. To answer this question, isolated *COR15a*-overexpressing protoplasts were frozen and the effects on the membranes were determined (Steponkus et al, 1998). A deferment in the production of hexagonal-II phase transitions was observed in protoplasts from COR15a overexpressing plants (Steponkus et al, 1998). The inner chloroplast membrane may be the most sensitive to freezing damage (Steponkus et al, 1998). If so, protecting this membrane, the "weak link" of plant membranes, would result in an overall increase in freezing tolerance in the plant. This raises the intriguing possibility that the COR15a polypeptide functions in association with the other COR polypeptides in an additive manner to protect plant membranes from freezing damage. However, whether this is the case for the other COR polypeptides remains to be determined.

1.4.6.5 COR gene regulation

COR gene transcript levels increase within about four h of a low temperature stimulus and remain high for as long as plants are kept at low temperatures (Hajela et al, 1990). This raised the question of how the *COR* genes are regulated. Promoter analysis of the *COR* genes lead to the discovery that they are transcriptionally regulated through a cold- and drought inducible promoter element, called the CRT/DRE (C-Repeat/Drought Responsive Element). The DRE element, TACCGACAT, was first identified by

Yamaguchi-Shinozaki and Shinozaki (1994) as being sufficient for drought and cold inducibility. The core of the DRE, CCGAC, called the CRT, is present in multiple copies in the *COR6.6* (Wang et al, 1995), *COR15* (Baker et al, 1994) and *COR78* (Horvath et al, 1993; Yamaguchi-Shinozaki and Shinozaki, 1993) promoters. Deletion experiments using the *COR15a* promoter showed that cold-inducible reporter gene activation was consistent with the presence of the CRT (Baker et al, 1994). Interestingly, further research has shown that this sequence is not only found in multiple copies in *COR* gene promoters in Arabidopsis, but also in the promoters of some of the cold induced genes in other species. These include the *BN115* gene in *Brassica napus* (Jiang et al, 1996), and the *wcs120* gene in wheat (Ouellet et al, 1998). The presence of *COR* gene homologues along with the conservation of the cold and drought inducible promoter element, the CRT/DRE, between diverse species strongly suggests that cold and drought inducible gene expression is highly conserved.

1.4.6.6 The CBF family of transcription factors

The CRT/DRE sequence is involved in cold- and drought-induced activation of the *COR* genes. The next question became, what protein was binding to the CRT/DRE and activating transcription under acclimating conditions? A major breakthrough in answering this question was the isolation of *CBF1*, (CRT/DRE binding factor 1) (Stockinger et al, 1997). *CBF1* was isolated in a one-hybrid assay using the *COR15a* CRT/DRE promoter element as bait. CBF1 protein contains a putative nuclear localization signal, (Raikhel, 1992) an AP2-like DNA binding domain (Weigel, 1995;

Ohme-Takagi and Shinshi, 1995) and an acidic C-terminal domain consistent with some types of transcriptional activators (Hahn, 1993). *In vitro* assays indicate that CBF1 binds specifically to the CRT/DRE sequence and *in vivo* assays demonstrate that CBF1 can activate transcription of a reporter gene under the control of the *COR15a* promoter in yeast (Stockinger et al, 1997).

Additional research has led to the discovery that *CBF1* is part of a small gene family consisting of *CBF1*, *CBF2* and *CBF3*, also called *DREB1b*, *DREB1c* and *DREB1a* respectively (Gilmour et al, 1998; Liu et al, 1998). The three *CBF* genes are highly similar in amino acid sequence (~85%) and are located in tandem about two kb apart on chromosome 4 (Gilmour et al, 1998). Like *CBF1*, *CBF2* and *CBF3* contain AP2-like DNA binding domains, putative acidic activation domains, and can activate transcription in yeast (Stockinger et al, 1997; Gilmour et al, 1998; Liu et al, 1998; Shinwari et al, 1998). All three CBF proteins can bind to CRT/DRE sequences contained within the *COR15a*, *COR15b*, and *COR78* promoters, and appear to have the highest binding affinity for the *COR15*b promoter (Gilmour et al, 1998). The reason for this preferential binding to the *COR15b* promoter is not currently known.

1.4.6.7 CBF *gene regulation*

RNA hybridization analysis has indicated that all three *CBF* transcripts are upregulated within 15 min of a low temperature stimulus, peak at two-four h, and remain at a higher steady state level for up to 24 hours (Gilmour et al, 1998). As seen previously, *COR* gene transcripts increase after ~four h suggesting the possibility of a cold induced

signal cascade (Gilmour et al, 1998). *CBF* transcripts are also induced by mechanical agitation, although only transiently. Whether the *CBF* transcripts are induced by ABA or osmotic stress has been difficult to determine accurately due to the low levels of RNA accumulation, although it appears a slight increase may occur (Gilmour et al, 1998; D. Zarka and M. Thomashow, unpublished).

Investigation of the promoters of the three *CBF* genes has shown that none contain CRT/DRE sequences (Gilmour et al, 1998). Additionally, overexpression of *CBF1* did not result in the activation of *CBF3*, strongly suggesting that the genes are not induced by autoregulation (Gilmour et al, 1998). While the promoters of the *CBF* genes contain consensus sequences consistent with G-box core and myc recognition sites, they do not contain known consensus sequences associated with cold activation (Gilmour et al, 1998). The exact mechanism by which the *CBF* genes are upregulated under cold-acclimating conditions remains to be determined.

1.4.6.8 The SFR6 mutant

One possible clue to how the CBF proteins function to regulate the *COR* genes comes from analysis of mutant Arabidopsis plants. Warren and colleagues (1996) screened for Arabidopsis mutants, *sfr* (sensitive to freezing), that had reduced freezing tolerance even after cold acclimation. One of the mutants, *sfr6,* has the interesting phenotype of an increase in *CBF* RNA accumulation under acclimating conditions, but no induction of *COR* gene expression (Knight et al, 1999). ABA and osmotic stress induced *COR* gene expression, as seen by assaying for *KIN1* (*COR6.6*) RNA

accumulation, is also lost. Cloning the gene containing this recessive mutation should give important clues as to how the CBF proteins activate *COR* gene expression.

1.4.6.9 Changes in calcium

Exposure to low temperature results in a transient increase in cytosolic calcium in plants. The rise is initiated by calcium influx through the plasmalemma and by release of calcium from internal stores, such as the vacuole (Knight et al, 1996). This change in calcium has been implicated in the control of cold-responsive gene expression (Knight et al, 1996; Tähtiharju et al, 1997) and in increasing freezing tolerance in plants (Monroy et al, 1993; Monroy and Dhindsa, 1995). Specifically, if chickpea plants are exposed to 0.5 mM $CaCl_2$, genes that are responsive to low temperature as well as other osmotic stresses are upregulated (Colorado et al, 1994). Work with alfalfa cell suspension cultures has shown that the cold inducibility of the *cas* genes, *cas15* and *cas18*, is obliterated when external calcium influxes are blocked with inhibitors, and that the genes are induced at 25° C when calcium ionophores or calcium channel agonists are added (Monroy and Dhindsa, 1995).

How calcium influxes specifically function to elicit cold-induced gene expression remains unknown. There is evidence that protein phosphorylation occurs after cold induced calcium influxes (Monroy et al, 1998). These phosphorylation events could be activated by calcium dependent protein kinases (CDPKs), or other types of protein kinases, such as mitogen-activated protein (MAP) kinases. At present, there is evidence that RNA accumulation of CDPKs in Arabidopsis increases after exposure to low

temperatures (Tähtiharju et al, 1997). The sequencing of the Arabidopsis genome has led Harmon et al (2000) to predict that 40 CDPKs are present in Arabidopsis.

There is evidence that cold- and osmotic stress-inducible MAP kinases exist in plants. Jonak et al (1996) have identified a MAP kinase in alfalfa, the activity of which is activated within 10 min of exposure of plants to low temperatures. Additionally, a MAP kinase and a MAP kinase kinase kinase have been identified in Arabidopsis, which are induced by touch, cold and water stress (Mizoguchi et al, 1996). Another kinase identified in Arabidopsis, ATMEKK1, is induced within 5 min of osmotic (NaCl) stress and increases the survival of yeast expressing ATMEKK1 under high NaCl conditions (Covic et al, 1999). Future work involving the characterization of these and other cold and stress-induced kinases should increase our understanding of the upstream signals involved in cold induction pathways.

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2. CHAPTER 2: Overexpression of Arabidopsis CBF1 results in increased COR gene expression and freezing tolerance without a low temperature stimulus

2.1 INTRODUCTION

The adverse environmental stress of freezing temperatures severely limits crop yield and geographical area of production (Levitt, 1980; Boyer, 1982). Any increase in the ability of plants to withstand low and freezing temperatures would therefore be of great economic importance. One way in which some plant species survive freezing temperatures is by cold acclimation, the process by which plants increase in freezing tolerance after exposure to low, nonfreezing temperatures. Understanding the various and complex processes involved in cold acclimation has long been the focus of research efforts as a potential means to increase freezing tolerance.

An early hypothesis was that cold acclimation, in addition to being associated with numerous physiological and biochemical changes, was associated with changes in gene expression (Weiser, 1970). This hypothesis was first conclusively demonstrated in spinach, (Guy et al, 1985) then extended in Arabidopsis with the isolation of a family of genes, called the *COR* genes (cold-regulated) -also called *LTI* (low temperature-induced), *KIN* (cold-inducible), *RD* (responsive to desiccation) and *ERD* (early dehydration-inducible)- which are greatly upregulated under cold-acclimating and drought conditions as well as by application of ABA (Hajela et al, 1990; Nordin et al, 1991; Wang et al,

1994; Welin et al, 1994). Analysis of the *COR* genes, which include *COR6.6, COR15, COR47* and *COR78*, has shown that all four *COR* genes are actually members of gene pairs, each of which is located in tandem in the genome (Thomashow et al, 1997). The proteins encoded by these genes are all highly hydrophilic and have the unusual trait of being soluble upon boiling in aqueous buffer (Lin et al, 1990; Thomashow, 1993). Amino acid analysis comparisons indicate that COR78, COR15 and COR6.6 are novel polypeptides while COR47 has similarities to LEA type II proteins, also known as dehydrins, which are associated with desiccation and drought (Dure et al, 1989; Gilmour et al, 1991; Thomashow et al, 1993; Close, 1997; Thomashow, 1999).

Due to their association with cold acclimation and drought conditions, both of which cause damage to membranes, and their similarities to LEA proteins, it has been hypothesized that the role of the *COR* genes is to protect membranes from osmotic stress damage. Work by Steponkus and colleagues has shown that the primary site of damage to plants during freezing conditions are the cellular membranes which experience freeze-induced dehydration (Steponkus et al, 1993; Steponkus, 1984). To identify a functional role for the COR15am protein, which is targeted to the chloroplast, Artus et al. (1996) generated *COR15a* overexpressing plants which were assayed for freezing tolerance. Compared to wild type plants no increase in freezing tolerance was seen at the whole plant level, however, an increase in the freezing tolerance of chloroplasts between -4 and -8° C was observed (Artus et al, 1996). When protoplasts overexpressing *COR15a* were investigated under freezing conditions, Steponkus et al (1998) found that there was a deferment of hexagonal II phase transitions to lower temperatures (see section 1.3), proposed to be due to changes in the intrinsic curvature of the inner membrane of the

chloroplast envelope (see section 1.4.6.4). Together, these data indicate a role for COR15 in protecting chloroplast membranes. Additionally, they present the intriguing possibility that the other *COR* genes may play similar roles in protecting plant membranes, and that the combined expression of all of the *COR* genes together results in the increase in freezing tolerance seen after cold acclimation. If so, then overexpression of the battery of *COR* genes (meaning all genes that are upregulated under acclimating conditions, not just the identified four *COR* gene families mentioned above) could result in increased freezing tolerance, as overexpression of one *COR* gene alone, *COR15*, did not.

In order to be able to manipulate the expression of all of the COR genes simultaneously, a better understanding *COR* gene regulation during cold acclimation was required. Promoter analysis led to the discovery that the *COR* genes are transcriptionally regulated through a cold- and drought inducible promoter element, called the CRT/DRE (C-Repeat/Drought Responsive Element). The DRE, TACCGACAT, was first identified by Yamaguchi-Shinozaki and Shinozaki (1994) as being cold and drought responsive. The core of the DRE, the CRT, CCGAC, is present in multiple copies within the promoters of *COR6.6* (Wang et al, 1995), *COR15* (Baker et al, 1994) and *COR78* (Horvath et al, 1993; Yamaguchi-Shinozaki and Shinozaki, 1993). Deletion experiments with the *COR15a* promoter showed reporter gene activation consistent with the CRT being the cis-acting acting element responsible for the cold-inducible activation (Baker et al, 1994). This element, however, is not associated with the ABA-induced activation of the *COR* genes (Baker et al, 1994; Yamaguchi-Shinozaki and Shinozaki, 1994; Wang et al, 1995). This observation corroborates the data indicating that ABA-induced activation of the *COR* genes occurs through a pathway separate from cold-and drought-induced

activation (Gilmour and Thomashow, 1991; Nordin et al, 1991) (see section 1.4.2).

A major breakthrough in understanding the signaling cascade activated by low temperature stimulus was the isolation of a transcription factor, called *CBF1* (CRT/DRE Binding Factor 1) (Stockinger et al, 1997). *CBF1* was suggested to be a unique gene or a member of a small gene family the protein product of which binds to the CRT/DRE element and activates transcription under cold acclimating conditions (Stockinger et al, 1997). The CBF1 protein contains an AP2-like DNA binding domain (Weigel, 1995; Ohme-Takagi and Shinshi, 1995), an acidic C-terminal portion that could act as a transcription activation domain (Hahn, 1993), and a putative nuclear localization domain (Raikhel, 1992), all of which are consistent with the conclusion that *CBF1* encodes a transcription factor. Gel shift experiments showed that CBF1 binds specifically to the CRT/DRE sequences contained within the *COR15a* promoter *in vitro* (Stockinger et al, 1997). Additionally, *in vivo* transcription activation assays showed that CBF1 can activate the transcription of a reporter gene under the control of the *COR15a* promoter in yeast (Stockinger et al, 1997). *CBF1* itself is upregulated under acclimating conditions in Arabidopsis (Gilmour et al, 1998). However, there do not appear to be any CRT/DRE sequences in the *CBF1* promoter, indicating that *CBF1* is not autoregulated (S. Gilmour and M. Thomashow, unpublished).

Here I present data indicating that CBF1 binds to the CRT/DRE and activates *COR* gene transcription in Arabidopsis plants. Transgenic Arabidopsis var. RLD plants that constitutively overexpress *CBF1* were generated. The transgenic plants have increased *COR6.6*, *COR15*, *COR47* and *COR78* RNA accumulation and increased COR6.6 and COR15 protein accumulation under nonacclimating conditions as compared

to wild type plants. Furthermore, the *CBF1*-overexpressing plants showed increased freezing tolerance as determined by electrolyte leakage assays and whole plant freeze tests. *CBF1* is therefore a likely activator of *COR* genes under acclimating conditions. Additionally the *COR* genes appear to play direct roles in increasing freezing tolerance as overexpression of the *COR* genes results in increased freezing tolerance without a low temperature stimulus. An attempt was also made to determine if *CBF1*-overexpressing plants also have increased tolerance to osmotic stress. However, no differences in germination frequency were seen between transgenic plants and control plants grown under conditions of osmotic stress.

Additionally, constitutive overexpression of *CBF1* was found to result in transgene silencing. Expression levels of the *CBF*-transgene decreased as the plants were self-pollinated and carried to the T3 –T5 generations. The reduction in transgene expression was closely correlated with a reduction in *COR* gene expression and a loss of increased freezing tolerance. In an attempt to determine the effects of eliminating *CBF1* expression, plants that constitutively overexpress *CBF1* in the antisense orientation were generated. Although RNA in the antisense orientation was present in transgenic plants, no differences in *COR* gene expression at either the RNA or proteins levels were detected.

2.2 MATERIALS AND METHODS

2.2.1 PLANT GROWTH

Arabidopsis thaliana (Arabidopsis) ecotype RLD plants were grown in pots under

100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ continuous light for 18-25 days as described (Gilmour et al, 1988). For experiments involving acclimation, plants were cold acclimated at 4° C under 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ continuous fluorescent illumination for the amount of time indicated. The types of plants used in experiments include wild type plants, RLD, plants that constitutively overexpress *COR15a*, T8 (Artus et al, 1996) and plants that constitutively overexpress CBF1, A6, B16, K16 and 1-11 (described below) in the T2-T5 generations.

2.2.2 PLANT TRANSFORMATION

Standard procedures were used for plasmid manipulations (Sambrook et al, 1989). Specifically, to make transgenic plants, the *CBF1*-containing *Ase*I-*Bgl* II fragment from pACT-Bgl+ (Stockinger, 1997) was gel purified, end-filled by Klenow (GibcoBRL, Grand Island, NY) and *Bam*HI linkers were ligated to both ends. The fragment was then digested with *Bam*HI and ligated into the *Bam*HI site of pCIB710 (Rothstein et al, 1987) which contains the Cauliflower Mosaic Virus (CaMV) 35S constitutive RNA promoter and terminator. Plasmids containing both the sense (pKJO1) and antisense (pKJO2) orientation of the *CBF1* gene were isolated and used to make constructs in both orientations. The chimeric plasmids were individually linearized at the *Kpn*I site and inserted into the *Kpn*I site of the binary vector pCIB10g (Ciba-Geigy, Research Triangle Park, NC), resulting in pKJO1a and pKJO2a for the sense and antisense constructs respectively. The plasmids were transformed into *Agrobacterium tumefaciens* strain C58C1 (pMP90) by electroporation. Arabidopsis plants were transformed by the vacuum

infiltration procedure (Bechtold, et al, 1993) as modified by van Hoof and Green (1996). Transgenic plants were selected by survival on 1 x Gamborgs B-5 media (GibcoBRL, Grand Island, NY) containing 50 μg/ml kanamycin. Homozygous lines of individual plants that had uniform kanamycin resistance in the T3 generation and high transgene expression were selected for further study (A6, B16, 1-11, and K16).

2.2.3 DNA HYBRIDIZATION

Total Arabidopsis DNA was isolated as described (Rogers and Bendich, 1988). Approximately 5-10 μg of total DNA was individually digested with *Hind*III or *Eco*RV, electrophoreased on 1% agarose gels, and transferred to nylon membranes (Micron Separations, Westboro, MA) following standard protocols (Sambrook et al, 1989). The high and low stringency conditions used were identical to those used for RNA accumulation analysis with the exception that the high-stringency wash was conducted at 65° C instead of 50° C (Stockinger et al, 1997). The DNA probe for *CBF1* (Stockinger et al, 1997) was gel purified full length *CBF1* labeled with ^{32}P by random-priming according to standard procedures (Sambrook et al, 1989).

2.2.4 RNA HYBRIDIZATION

Total RNA was isolated from plant leaves as described (Gilmour et al, 1988). Northern transfers were prepared using 20 μg of total RNA electrophoreased in 1.5%

formaldehyde gels. The resulting membranes were hybridized to ^{32}P labeled double stranded DNA probes and washed at high stringency (Stockinger et al, 1997). Double stranded DNA probes for *CBF1* (Stockinger et al, 1997), *COR6.6, COR15, COR47, COR78* (Hajela et al, 1990) and *EIF4A* (Metz et al, 1992) were gel purified and labeled with ^{32}P by random-priming according to standard procedures (Sambrook et al, 1989).

2.2.5 IMMUNOBLOT ANALYSIS

Total soluble protein was extracted by grinding frozen tissue (about 150 mg) in 400 µl extraction buffer containing 50 mM EDTA (pH 8.0), 50 mM HEPES (pH 7.0), and 1.5% (wt/vol) polyvinyl-pyrrolidone, after which insoluble material was removed by centrifugation (13,000 x g for 20 min at 4° C). The protein concentration in the supernatant was determined using the Bradford dye-binding assay (Bio-Rad, Hercules, CA). Total soluble protein (100 µg per sample) was fractionated by 10% tricine SDS/PAGE (Schägger and von Jagow, 1987), and transferred to 0.2 µm nitrocellulose membranes by electroblotting (Towbin et al, 1979) as described (Artus et al, 1996). COR15 protein was detected using antiserum raised to purified COR15am (Artus et al, 1996) and protein A conjugated to alkaline phosphatase (Sigma) as described (Blake et al, 1984). No reacting bands were observed with preimmune serum.

2.2.6 ELECTROLYTE LEAKAGE ASSAYS

Electrolyte leakage assays were conducted as described (Sukumaran and Weiser, 1972; Gilmour et al, 1988) with the following modifications. Five replicates of two to four detached leaves from nonacclimated or cold-acclimated plants were placed in a test tube and submerged in a –2° C bath containing water and ethylene glycol in a completely randomized design for 1 h. Ice crystals were added to nucleate freezing. After an additional h at –2° C, the samples were cooled in decrements of 1° C each h until –8° C was reached. Samples (five replicates of each data point) were thawed overnight on ice and shaken in 3 ml distilled water at room temperature for 3 h. Electrolyte leakage from leaves was measured with a conductivity meter. The solution was then removed, the leaves were frozen at –80° C for 1 to 10 h. The solution was returned to each tube and shaken for another 3 h to obtain a value for 100% electrolyte leakage.

The temperature at which 50% of electrolytes were leaked (EL_{50}) were determined by fitting model curves of up to third-order linear polynomials for each electrolyte leakage test. To ensure unbiased predictions of electrolyte leakage, trends significantly improving the model fit at the 0.2 probability level were retained. EL_{50} values were calculated from the fitted models. An unbalanced one-way analysis of variance (ANOVA), adjusted for the different number of EL_{50} for each type was determined using SAS PROC GLM [SAS Institute, SAS/STAT User's Guide, Version 6 (SAS Institute, Cary, NC, 1989)].

2.2.7 WHOLE PLANT FREEZE TESTS

Nine-centimeter pots containing ~40 RLD, T8 (*COR15a*-overexpressing) B16 or A6 (*CBF1*-overexpressing) Arabidopsis plants that were either 20 days old and nonacclimated or 25 days old and 4-day cold-acclimated plants were placed in a completely randomized design in a –5° C cold chamber in the dark. After 1 h, ice chips were added to each pot to nucleate freezing. Plants were removed after 1-3 days, returned to a growth chamber at 22° C and allowed to recover for 7 days before photographs were taken.

2.2.8 SALT STRESS GERMINATION TESTS

Wild type RLD plants and two *CBF1*-overexpressing transgenic lines in the T_3 generation, A6 and B16, were germinated on petri plates containing 1 x Gamborgs B-5 media (GibcoBRL,Grand Island, NY) solidified with 1% agarose, or Gamborgs B-5 media, 1% agarose and 75, 100, 125, 150 or 175 mM NaCl (Saleki, 1993). Approximately 100-200 seeds suspended in a 1 ml 0.1% agarose solution were plated onto each of the 6 types of plates. All plates were observed for germination/plant growth for 2 weeks.

2.3 RESULTS

2.3.1 ISOLATION OF CBF1*-OVEREXPRESSING ARABIDOPSIS PLANTS*

Plants that overexpress *CBF1* under the control of the 35S constitutive CaMV

promoter were created by transforming RLD plants with pKJO1a as described (see section 2.2.2). Transgenic plants were isolated by selecting for kanamycin resistance on media containing 50 μg/l kanamycin. T2 generation seeds from independent T1 plants were pooled and screened for *CBF1* RNA accumulation under nonacclimating conditions and after four h of cold-acclimation (data not shown). Initial screening identified two lines that had increased *CBF1* RNA accumulation under nonacclimating conditions, A6 and B16. Southern hybridization analysis using DNA from plants in the T3 generation indicated that A6 had T-DNA inserted at a single locus while B16 contained multiple T-DNA inserts (see Figure 2.1).

2.3.2 Overexpression of CBF1 *in Arabidopsis results in increased* COR *gene expression*

Examination of the A6 and B16 transgenic lines in the T_4 generation indicates that these lines have increased *CBF1* RNA accumulation under nonacclimating conditions as compared to wild type plants (Figure 2.2A). To determine if the increased accumulation of *CBF1* RNA resulted in increased *COR* RNA accumulation, northern hybridization analysis was performed using *COR6.6*, *COR15*, *COR47* and *COR78* as probes (Figure 2.2A). Overexpression of *CBF1* clearly results in the activation of *COR* gene transcription as increased *COR6.6*, *COR15*, *COR47* and *COR78* RNA accumulation

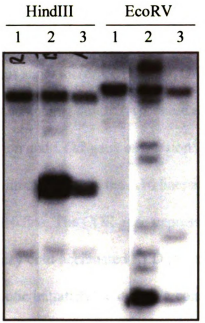

Figure 2.1. Southern hybridization of *CBF1*-overexpressing lines A6 and B16

Southern hybridization of wild type and transgenic DNA. Southern transfers of RLD, A6 and B16 T3 generation DNA that had been digested with HindIII and EcoRV were hybridized at high stringency using the entire *CBF1* coding region as described in 2.2.4. HindIII cuts out the *CBF1* cDNA fragment from the binary vector and EcoRV cuts once within the *CBF1* cDNA and once within the binary vector. Lanes are as follows:
1: RLD;
2: B16;
3: A6.

occurs in the *CBF1*-overexpressing lines as compared to wild type under nonacclimating conditions. A *COR15a*-overexpressing line, T8 is also included as a control (Artus et al, 1996) (Figure 2.2A). As would be expected, T8 shows an increase in only *COR15* RNA accumulation and does not show increased *CBF1, COR6.6, COR47* or *COR78* RNA accumulation under nonacclimating conditions. There appears to be a correlation between *CBF1* transgene expression and *COR* gene expression. The A6 line, which has higher levels of *CBF1* RNA accumulation than the B16 line, also has higher levels of *COR* RNA accumulation than the B16 line. The *COR* gene expression levels in the A6 line appear to approximate those of 3-day cold-acclimated RLD plants. Overexpression of *CBF1* has no effect on *eIF4A*, (eukaryotic initiation factor 4A) a constitutively expressed gene that is not involved with cold acclimation (Metz et al, 1992) and is therefore used as a loading control.

To determine if increased *COR* RNA results in increased COR protein accumulation, immunoblot analyses were conducted with antisera raised to COR15 (Figure 2.2B) and COR 6.6 (data not shown). As was observed for RNA accumulation, the nonacclimated A6 and B16 lines have increased protein accumulation as compared to nonacclimated RLD plants. Consistent with the RNA accumulation patterns, nonacclimated A6 plants have greater levels of COR protein accumulation than nonacclimated B16 plants. The COR15 protein accumulation in A6 is similar to that of seven-day cold acclimated RLD plants while the nonacclimated B16 plants have COR15 protein accumulation similar to that of four-day cold-acclimated RLD plants.

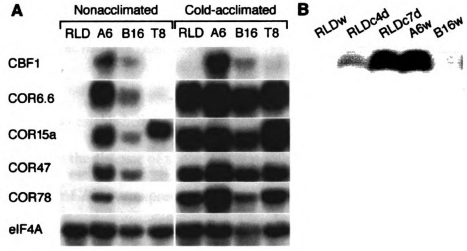

Figure 2.2. Expression of *CBF1* and *COR* genes in wild type and transgenic Arabidopsis plants

A. *CBF1* and *COR* gene RNA accumulation. The amount of *CBF1* and *COR* gene RNA accumulation in leaves of 3-day cold acclimated, and nonacclimated RLD, *CBF1*-overexpressing A6 or B16 and *COR15a*-overexpressing T8 plants was determined as described in 2.2.4. *eIF4A* (eukaryotic initiation factor 4a) is included as a loading control..

B. COR15m protein accumulation. The amount of COR15m accumulation in nonacclimated RLD, A6 and B16 leaves (RLDw, A6w, B16w) and 4- and 7-day cold-acclimated RLD plants (RLDc4d and RLDc7d respectively) was determined as described in 2.2.5

2.3.3 *OVEREXPRESSION OF* CBF1 *RESULTS IN INCREASED FREEZING TOLERANCE AS DETERMINED BY ELECTROLYTE LEAKAGE ANALYSIS*

Once it was established that overexpression of *CBF1* results in increased *COR* gene expression in the absence of a low temperature stimulus, the next step was to determine whether *CBF* overexpression also results in increased freezing tolerance. To do this, electrolyte leakage assays were conducted (see 2.2.6). By freezing leaves to various sub-zero temperatures then thawing them, the amount of cellular damage that has occurred due to freeze-induced membrane lesions can be assayed by measuring the leakage of electrolytes from the cells with a conductivity meter. Figure 2.3 shows the results of two individual electrolyte leakage tests. Figure 2.3A and B both show the graphical representation of the percentage of total electrolytes leaked by nonacclimated RLD, *CBF1*-overexpressing (A6 and B16), *COR15a*-overexpressing (T8) and 7 and10-day cold-acclimated RLD plants. Figure 2.3A shows nonacclimated RLD, A6, B16, and 10-day cold-acclimated RLD plants from 0 to –8° C . Under nonacclimating conditions, the A6 line has significantly less leakage than RLD plants from –4 to –8° C, and the B16 line has significantly less leakage than RLD plants from –5 to –7° C ($P < .001$). Figure 2.3B shows nonacclimated RLD, A6, T8, and 7-day cold-acclimated RLD plants from 0 to -8° C. While the nonacclimated A6 line has significantly less leakage than the nonacclimated RLD plants from –3 to –8° C ($P < .003$) the T8 line is not significantly different from nonacclimated RLD plants at any of the temperatures tested.

In order to combine the results of multiple electrolyte leakage tests, the

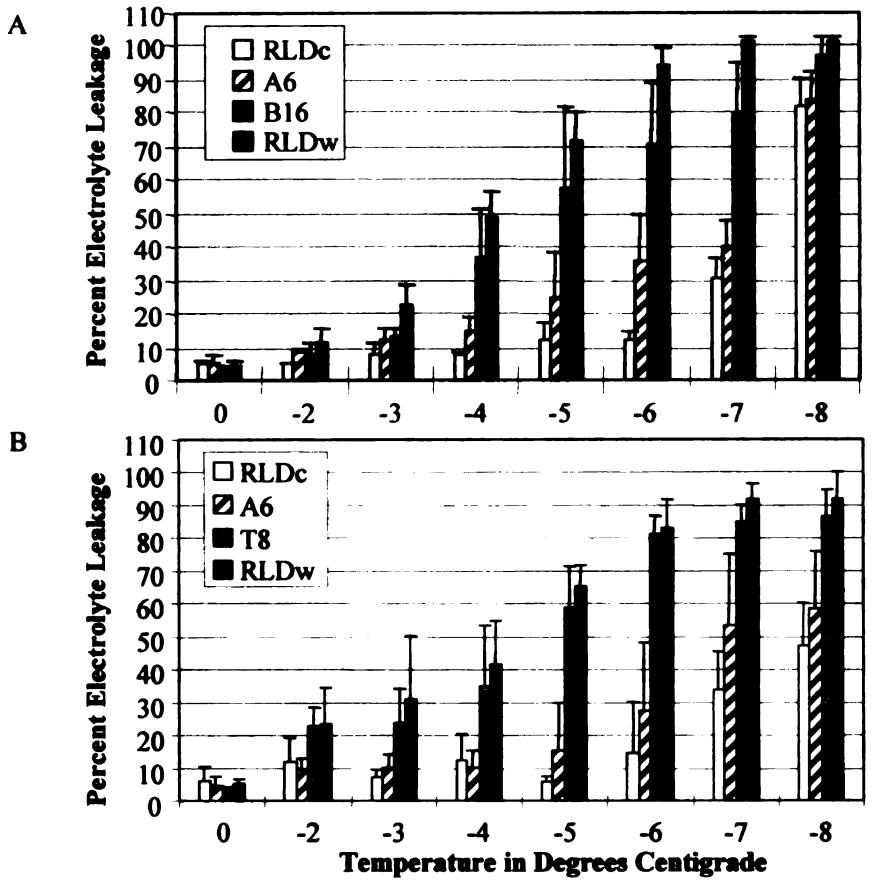

Figure 2.3. Electrolyte leakage analysis of wild type and transgenic Arabidopsis plants.

Leaves from nonacclimated RLD (RLDw), ten-day cold-acclimated RLD (RLDc) and nonacclimated *CBF1*-overexpressing A6 and B16 or *COR15a*-overexpressing plants were frozen at the indicated temperatures and the extent of cellular damage was estimated by measuring electrolyte leakage as described in 2.2.6. Error bars indicate standard deviations of the five replicates of each data point.

A. Electrolyte leakage analysis of ten-day cold-acclimated RLD (RLDc) and nonacclimated A6, B16, or RLD plants (RLDw) as described above.

B. Electrolyte leakage analysis of ten-day cold-acclimated RLD (RLDc) and nonacclimated A6, T8 or RLD plants (RLDw) as described above.

temperature at which 50% leakage occurs (EL_{50}) in all electrolyte leakage tests conducted was estimated (see Table 2.1). These data indicate that the *CBF1*-overexpressing transgenic lines A6 and B16 have a statistically significant increase in freezing tolerance as compared to RLD plants under nonacclimating conditions. In fact, B16 has a ~1.3° C increase in freezing tolerance and A6 has a ~3.2° C increase in freezing tolerance compared to RLD plants under nonacclimating conditions. As seen previously, the *COR15a*-overexpressing line T8 does not have a significant increase in freezing tolerance compared to control plants (Artus et al, 1996). Interestingly, the EL_{50} of nonacclimated A6 plants is not significantly different from the EL_{50} of 7-10 day cold-acclimated RLD plants while both B16 and T8 have an EL_{50} that is significantly different from that of 7-10 day cold-acclimated RLD plants. These data indicate that overexpression of one *COR* gene, *COR15*, does not result in a significant increase in freezing tolerance but that overexpression of the battery of *COR* genes, as seen in the A6 and B16 lines, does result in a significant increase in freezing tolerance.

2.3.4 OVEREXPRESSION OF CBF1 *RESULTS IN INCREASED FREEZING TOLERANCE AS SEEN BY WHOLE PLANT FREEZE TESTS, BUT NO GROSS PHENOTYPIC CHANGES*

Electrolyte leakage tests are an excellent way to approximate the freezing tolerance of plants. Another biologically significant way to determine freezing tolerance is to freeze whole plants and score for survival. Pots of nonacclimated RLD, T8, A6, and B16 plants along with 5-to 10- day cold-acclimated RLD plants were frozen to –5° C for 1-3 days, returned to the 22° C growth chamber and allowed to recover for 1-2 weeks. In all nine

Table 2.1. Comparison of Electrolyte leakage 50% (EL_{50}) values of leaves from wild type and transgenic Arabidopsis plants.

EL_{50} values					
	RLDw	RLDc	A6	B16	T8
RLDw	-3.9 ± 0.21 (8)	P < 0.0001	P < 0.0001	P = 0.0014	P = 0.7406
RLDc		-7.6 ± 0.30 (4)	P = 0.3261	P < 0.0001	P < 0.0001
A6			-7.2 ± 0.25 (6)	P < 0.0001	P < 0.0001
B16				-5.2 ± 0.27 (5)	P = 0.0044
T8					-3.8 ± 0.35 (3)

EL_{50} (the temperature at which 50% leakage occurred) values were calculated and compared by unbalanced one-way ANOVA using SAS PROC GLM as described in 2.2.6. EL_{50} values ± SE (n) are presented on the diagonal line for leaves from nonacclimated RLD (RLDw), 7-10 day cold-acclimated RLD (RLDc) plants, nonacclimated A6, B16 (*CBF1*-overexpressing), and T8 (*COR15a*-overexpressing) plants. P values for comparisons of the different EL_{50} values are indicated in the corresponding boxes.

replications of this experiment, the survival of plants from the B16 and T8 lines did not appear significantly different from that of nonacclimated RLD plants. However, the survival of A6 plants, while somewhat variable, was always greater than that of nonacclimated RLD plants. An example of a freeze test is shown in Figure 2.4. Nonacclimated RLD and A6 plants, and 5-day cold-acclimated RLD plants were frozen to –5° C for 2 days and then allowed to recover for 7 days at 22° C. The nonacclimated RLD plants did not survive being frozen whereas the A6 plants and the 5-day cold-acclimated RLD plants did. This clearly indicates that the overexpression of *CBF1* results in increased freezing tolerance in the absence of a low temperature stimulus as compared to wild type plants. There were no gross phenotypical differences seen between transgenic A6 and RLD plants (see Figure 2.4).

2.3.5 OVEREXPRESSION OF CBF1 *DOES NOT RESULT IN INCREASED GERMINATION UNDER OSMOTIC STRESS*

As freezing, drought, and osmotic stress are similar stresses in that they all result in dehydration (Thomashow, 1999), it was possible that *CBF1*-overexpressing plants also had increased tolerance to germinate under conditions of osmotic stress. The addition of NaCl to plant growth media results in osmotic stress. To test if *CBF1*-overexpressing plants have an increased ability to germinate under osmotic stress, T3 seeds from the two *CBF1*-overexpressing transgenic lines, A6 and B16 were germinated on media containing 0, 75, 100, 125, 150 or 175 mM NaCl as described in section 2.2.8 (see Table 2.2). After two weeks of observation, no differences in germination frequency could be seen

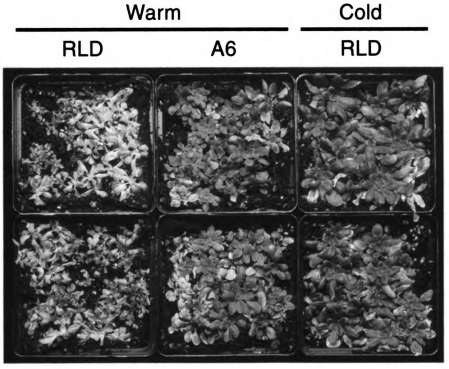

Figure 2.4. Freezing survival of RLD and *CBF1*-overexpressing A6 Arabidopsis plants.

Nonacclimated (Warm) RLD and A6 plants and 5-day cold acclimated (Cold) RLD plants were frozen at -5^0 C for 2 days and then returned to a growth chamber at 22^0 C as described in 2.2.7. A photograph of the plants after 7 days of recovery at 22^0 C is shown. This figure is shown in color.

between the transgenic lines and the RLD control lines. All three types of seed had 80-100% germination on NaCl concentrations of 125 mM or less and none of the seeds from RLD or the *CBF1*-overexpressing lines germinated at NaCl concentrations of 150 mM or higher.

2.3.6 Overexpression of CBF1 results in transgene silencing in all lines tested

All previous experiments described with the A6 and B16 transgenic lines were conducted on the T3 and T4 generations. However, starting in the T4 generation, a dramatic increase in the amount of electrolytes leaked between B16 plants in the T3 and T4 generations was observed (see Figure 2.5A). To determine what was causing the decrease in freezing tolerance between the T3 and T4 generations, *CBF1* RNA accumulation in the T3 and T4 generations of the B16 line was compared (see Figure 2.5B). Nonacclimated RLD and 5-day cold-acclimated RLD plants are also included as controls. There is less *CBF1* RNA accumulation in the T4 generation than in the T3 generation of the B16 line, and as would be expected, correlatively less *COR15* and *COR47* RNA accumulation in the T4 generation of B16 plants (see Figure 2.5B). A reduction in transgene expression was also seen with the *CBF1*-overexpressing line A6 in the T5 (see Figure 2.6). To understand better the cause of the reduction, individual families of A6 T5 plants were assayed (see Figure 2.6A). Sixteen individual A6 plants of the T4 generation were allowed to self-pollinate and T5 seed was collected separately for each plant. Members of each family of the individual T5 lines were bulked and tested for COR15m protein accumulation (see Figure 2.6A) due to difficulties detecting *CBF1*

Table 2.2. Germination of *CBF1*-overexpressing and wild type plants on NaCl

NaCl Concentration (in mM)	0	75	100	125	150	175
RLD	+	+	+	+	-	-
A6	+	+	+	+	-	-
B16	+	+	+	+	-	-

Seeds from RLD, A6 and B16 in the T3 generation were germinated the various levels of NaCl indicated above and observed for germination for two weeks as described in 2.2.8. The percentage of germination is indicated with a "+" or a "-" symbol. All plates that had 80 to 100% germination are indicated with a "+" and all plates that had 0% germination are indicated with a "-".

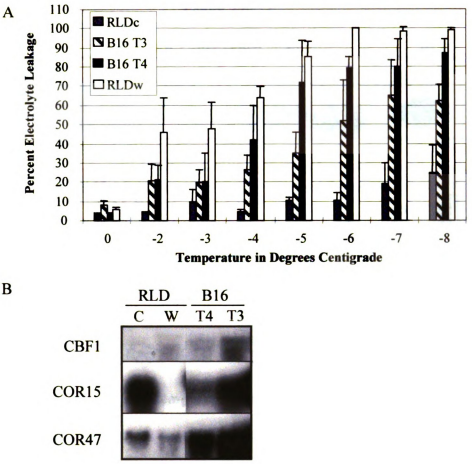

Figure 2.5. Electrolyte leakage analysis and RNA accumulation of wild type and B16 plants.

A. Freezing tolerance of leaves from RLD and B16 transgenic Arabidopsis plants. Leaves from nonacclimated RLD (RLDw), 10-day cold-acclimated RLD (RLDc), and nonacclimated B16 T3 and B16 T4 generation plants were frozen at the indicated temperatures and the extent of cellular damage was estimated by measuring electrolyte leakage as described in 2.2.6. Error bars indicate standard deviations of the five replicates of each data point.

B. CBF1 and COR RNA accumulation. CBF1 and COR gene RNA accumulation in leaves of five-day cold acclimated RLD plants (C), nonacclimated RLD (W) and B16 T3 and B16 T4 generation plants was determined as described in 2.2.4

Figure 2.6

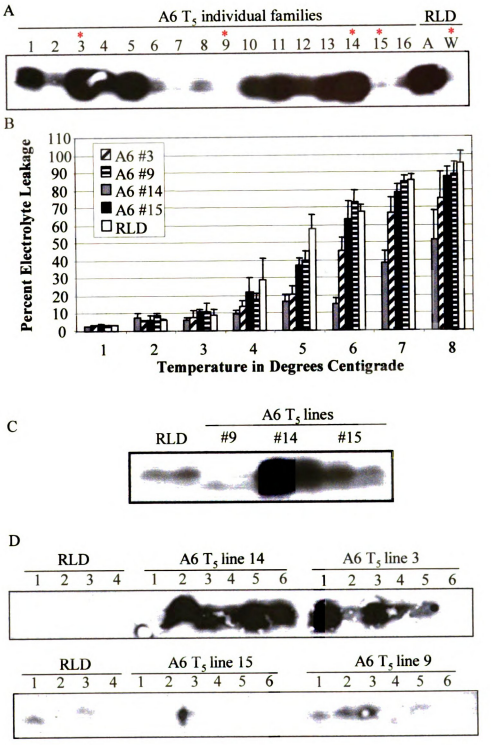
A
A6 T5 individual families
RLD
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 A W
B
Percent Electrolyte Leakage
110
100
90
80
70
60
50
40
30
20
10
0
A6 #3
A6 #9
A6 #14
A6 #15
RLD
1 2 3 4 5 6 7 8
Temperature in Degrees Centigrade
C
A6 T5 lines
RLD
#9
#14
#15
D
RLD
A6 T5 line 14
A6 T5 line 3
1 2 3 4 1 2 3 4 5 6 1 2 3 4 5 6
RLD
A6 T5 line 15
A6 T5 line 9
1 2 3 4 1 2 3 4 5 6 1 2 3 4 5 6

Figure 2.6 cont.

E

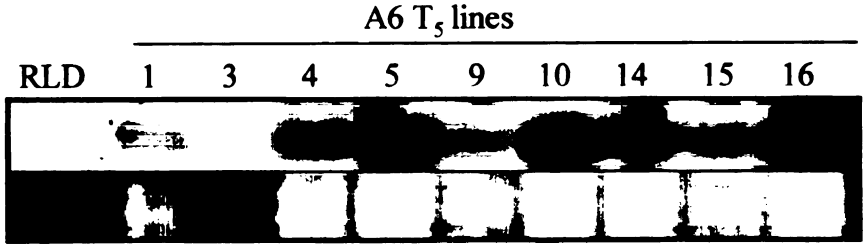

Figure 2.6. Reduced COR15m accumulation is associated with reduced freezing tolerance, but not the loss of the transgene in A6 T5 Lines

A. COR15m protein accumulation. Amount of COR15m protein accumulation in 6-day cold-acclimated RLD plants (RLDA), nonacclimated RLD plants (RLDW) and individual families of A6 T5 plants (A6 T5 1-16). The amounts of COR15m were determined as described in 2.2.5. A6 T5 families that were further investigated are indicated with an •.

B. Freezing tolerance of leaves from RLD and individual families of transgenic Arabidopsis plants. Leaves from nonacclimated RLD (RLD) and selected families of A6 T5 lines (A6 #3, A6 #9, A6 #14, A6 #15) were frozen at the indicated temperatures and the extent of cellular damage was estimated by measuring electrolyte leakage as described in 2.2.6. Error bars indicate standard deviations of the five replicates of each data point.

C. COR15m protein accumulation. The amount of COR15m protein accumulated in RLD and selected A6 T5 families (#9, #14, #15) after 7-days of cold-acclimation was determined for 2 individually isolated sets of protein as described in 2.2.5

D. COR15m protein accumulation. Amount of COR15m protein accumulated in nonacclimated leaves of four individual RLD plants and six individual plants of selected A6 T5 families, #3, #9, #14 and #15 as described in 2.2.5

E. Presence of the *CBF1*-transgene in selected A6 T5 families. Genomic DNA was isolated from individual families of A6 T5 plants (#1, #3, #4, #5, #9, #10, #14, #15, #16) and probed for the presence of the *CBF1*-transgene as described in 2.2.3. The *Hind*III- *CBF1* containing fragment is shown is shown in the upper panel. A portion of the ethidium bromide stained agarose gel containing genomic DNA to which the *CBF1* probe was hybridized is in the panel below

RNA accumulation (data not shown). COR15m protein accumulation varied greatly between the families, some of which had little or no expression (family # 2, 6, 7, 8, 9, 15 and 16) while others had intermediate or high levels of expression (family # 1, 3, 4, 5, 10, 11, 12, 13, and 14) as compared to nonacclimated RLD plants. Six-day cold acclimated RLD plants are also included as a control. To determine if there was a correlation between the low levels of COR15m protein accumulation and freezing tolerance, electrolyte leakage assays were conducted on two lines with low COR15m protein accumulation, #3 and #14, and two lines with high levels of COR15m protein accumulation, #9 and #15 (Figure 2.6B). There is a clear correlation between freezing tolerance and COR15m protein accumulation. The families with little or no COR15m protein accumulation, (#3 and #14) have similar electrolyte leakage percentages to nonacclimated RLD plants while the families with high levels of COR15m protein accumulation (#9 and #15) have lower electrolyte leakage percentages than nonacclimated RLD plants.

There were four possible reasons for the probable loss of *CBF1*-overexpression. 1) Co-suppression of the endogenous *CBF1* gene by the *CBF1* transgene had occurred resulting in a total loss of *CBF1* expression; 2) The individual plants in the lines with low expression had all independently lost *CBF1*-induced COR15 expression due to transgene silencing; 3) The individual plants had variable levels of transgene expression, but the majority of individual plants had lost expression due to transgene silencing; or 4) due to mislabeling of seed material, the *CBF1* transgene was not present in those lines.

To determine which hypothesis was correct, lines with little or no COR15m protein accumulation under nonacclimating conditions were acclimated and assayed for

COR15m accumulation. If co-suppression had occurred, COR15m protein accumulation should not be detected under acclimating conditions, assuming that *CBF1* was the only activator of *COR* gene expression under acclimating conditions (the existence of *CBF2* and *CBF3* was not known at the time the experiment was conducted). There was no reduction in COR15m protein accumulation in the A6 T5 generation transgenic lines #9, #14 and #15 compared to RLD plants acclimated for seven days suggesting that cosupression had not occurred (see Figure 2.6C).

To assay for the expression levels of individual plants, six individual plants from four selected lines, two with high COR15m protein accumulation (#3 and #14), two with low COR15m protein accumulation (#9 and #15), and four individual nonacclimated RLD plants were assayed for COR15m protein accumulation (see Figure 2.6D). Little or no COR15m protein accumulation is seen in individual plants from the two lines with low expression (#9 and #15), while variable but significant COR15m protein accumulation is seen in the two lines with high expression (#3 and #14) compared to the individual nonacclimated RLD plants. These data indicate that the expression level of COR15m protein was consistent within families, but not across the families.

To ensure that the low level of COR15m protein accumulation in lines with low expression was not due to loss of the *CBF1* transgene, Southern hybridization analysis was conducted (Figure 2.6E). The *Hind*III fragment of the binary plasmid which contains the *CBF1*-transgene was present in all nine A6 T5 families tested, and not present in non-transformed RLD plants. Variations in the intensity of the banding pattern are due to uneven loading. Combined, these data strongly suggest that loss of COR15m protein accumulation was due to transgene silencing and not due to the loss of the *CBF1*

transgene or cosuppression of all copies of the *CBF1* gene.

Two additional lines, showing increased *CBF1* RNA, or *COR15* RNA accumulation under nonacclimating conditions, were isolated, K16 (see Figure 2.7A) and 1-11 (see Figure 2.7B) respectively. In electrolyte leakage tests, T2 plants from the K16 line appeared to have equal or less leakage than 5-day cold-acclimated RLD plants (Figure 2.8A). However, both of these lines exhibited transgene silencing in the T3 generation. Figure 2.8B shows the results of an electrolyte leakage assay on K16 plants in the T3 generation. There is little difference between K16 plants and nonacclimated RLD plants indicating that transgene silencing has most likely occurred.

2.3.7 ANTISENSE CBF1 PLANTS *DO NOT HAVE REDUCED* CBF1 *OR* COR *GENE EXPRESSION*

One way to determine the effects of a gene is to delete it from the genome and test for any changes in phenotype. It was of interest to determine if there were any changes in the phenotype of plants that had total loss of *CBF1* gene expression. To test for this, plants were transformed with the antisense orientation of the *CBF1* gene under the control of the constitutive CaMV 35S promoter (see section 2.2.2) as overexpression of a gene in the antisense orientation can result in the loss of expression of the endogenous gene (van der Krol et al, 1990). A total of 15 independent transgenic lines in the T2 generation were screened for *CBF1* RNA accumulation under nonacclimating conditions, and *COR* RNA accumulation under cold-acclimating conditions using double stranded DNA probes. Figure 2.9A and B are examples of typical data. While there was increased *CBF1* RNA accumulation under nonacclimating conditions as determined by using a double stranded

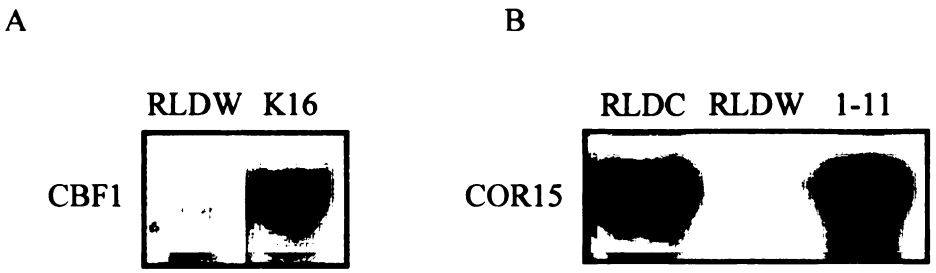

Figure 2.7. *CBF1* and *COR15* RNA accumulation in *CBF1*-overexpressing plants

CBF1 or *COR15a* RNA accumulation in leaves of 5-day cold-acclimated RLD (RLDC), nonacclimated (RLDW), K16 and 1-11 (*CBF1*-overexpressing) T2 generation plants was determined as described in 2.2.4.

A. *CBF1* RNA accumulation. CBF1 RNA accumulation in nonacclimated RLD and T2 generation K16.

B. *COR15* RNA accumulation. COR15 RNA accumulation in 5-day cold acclimated RLD, nonacclimated RLD and T2 generation 1-11.

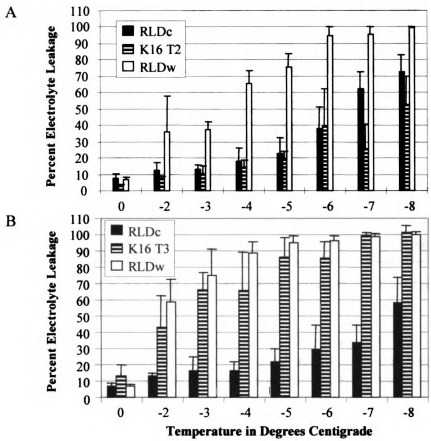

Figure 2.8. Electrolyte leakage analysis of wild type and transgenic K16 plants.

Leaves from nonacclimated RLD, cold-acclimated RLD K16 T2 and T3 generation plants were frozen at the indicated temperatures and the extent of cellular damage was estimated by measuring electrolyte leakage as described in 2.2.6. Error bars indicate standard deviations of the five replicates of each data point.

A. Electrolyte leakage analysis using five-day cold-acclimated RLD plants (RLDc) and nonacclimated K16 T2 (K16 T2) or RLD plants (RLDw) as described above.

B. Electrolyte leakage using analysis four-day cold-acclimated RLD plants (RLDc) and nonacclimated K16 T3 (K16 T3) or RLD plants (RLDw) as described above.

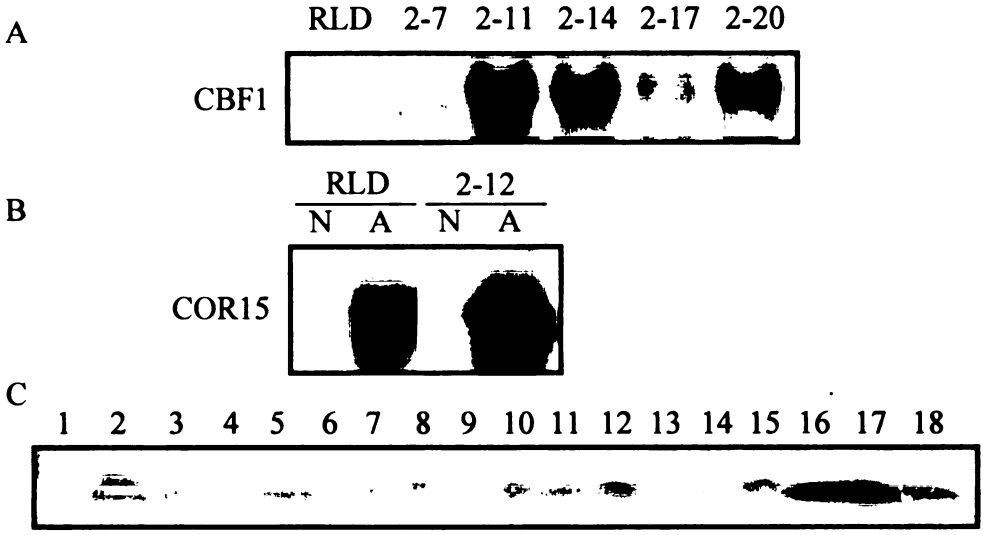

Figure 2.9. *CBF1* and *COR* gene accumulation in antisense-*CBF1* transgenic lines

CBF1 and *COR15* RNA accumulation. The amount of *CBF1* and *COR15* RNA accumulation in leaves of nonacclimated (N) and acclimated (A) RLD and antisense (AS) *CBF1*-overexpressing lines was determined as described in 2.2.4.

A. *CBF1* RNA accumulation. *CBF1* RNA accumulation in nonacclimated RLD and 5 independent lines overexpressing AS *CBF1* in the T2 generation using a double stranded probe. The AS *CBF1* transcript is approximately the same size as the endogenous *CBF1* transcript.

B. *COR15* RNA accumulation. *COR15* RNA accumulation in nonacclimated and 7-day cold acclimated RLD and 1 independent line overexpressing AS *CBF1* in the T3 generation.

C. COR15m protein accumulation. The amount of COR15m Protein accumulation in nonacclimated RLD (RLDW) and 7-day cold acclimated RLD (RLDC)and 17 independent lines overexpressing AS *CBF1* in the T2 generation was determined as described in 2.2.5. Lanes are as described below:

1: RLDW	5: 2-18.2	9: 2-20.1	13: 2-22.3	17: 2-24.3
2: RLDC	6: 2-18.3	10: 2-20.2	14: 2-22.4	18: 2-24.4
3: 2-17.2	7: 2-19.1	11: 2-22.1	15: 2-22.5	
4: 2-18.1	8: 2-19.2	12: 2-22.2	16: 2-24.1	

CBF1 cDNA probe Figure 2.9A), there were no dramatic differences seen between the amount of *COR15* RNA accumulation between antisense *CBF1* plants and RLD plants under acclimating conditions (Figure 2.9B). In fact, there appears to be a slight increase in *COR15* accumulation in antisense-CBF1 line 2-12 as compared to control plants (see Figure 2.9B). The reason for this apparent increase is not known.

An additional 32 T2 generation anti-sense *CBF1* lines were screened by assaying for COR15m protein accumulation under cold-acclimating condition, 17 of which are shown in Figure 2.9C. Again, no dramatic differences were seen between transgenic and RLD plants under acclimating conditions. Some of the same lines were again screened in the T3 generation for COR15m protein accumulation and identical results were seen (data not shown). These data indicate that either the endogenous *CBF1* had not been cosuppressed, or that cosuppression of *CBF1* does not result in a reduction of *COR* gene expression under acclimating conditions.

2.4 DISCUSSION

CBF1 was previously shown to bind to the CRT/DRE and activate transcription of a reporter gene in yeast (Stockinger, 1997). Here I present data indicating that CBF1 can also bind to the CRT/DRE and activate transcription of the *COR* genes in plants since overexpression of *CBF1* in Arabidopsis resulted in increased *COR* gene expression under nonacclimating conditions (see Figure 2.1 and Figure 2.2). Interestingly, increased activation of *COR* genes resulted in increased freezing tolerance as seen by both electrolyte leakage tests (see Figure 2.3, Figure 2.5A, Figure 2.6B and Figure 2.8A and

Table 2.1) and whole plant freeze tests (see Figure 2.4). These results strengthen the argument that the COR proteins play additive roles in increasing freezing tolerance during cold acclimation, as was previously suggested by overexpression of a single COR protein, COR15a (Artus et al, 1996).

The increase in *CBF1* RNA accumulation in the absence of a low temperature stimulus seen in *CBF*-overexpressing lines (see Figure 2.2) may provide clues as to the mechanisms of *CBF1* regulation. If *CBF1* was regulated by the rapid degradation of RNA under nonacclimating conditions, then it would expected that ectopic overexpression of *CBF1* under a constitutive promoter would result in low levels of *CBF1* RNA under nonacclimating conditions and very high levels under cold-acclimating conditions. As this was not the case, [there is a approximately two fold increase in *CBF1* RNA under acclimating conditions as compared to non-acclimating conditions (see Figure 2.2)], it appears that *CBF1* is not regulated by RNA degradation. However, the possibility cannot currently be ruled out that overexpression of *CBF1* results in an increase *CBF1* transcript to the extent that the degradation enzymes are "overwhelmed" under nonacclimating conditions and cannot degrade all the transcripts.

It is also interesting to note that, while under both nonacclimating and acclimating conditions, the A6 line appears to have greater amounts of *CBF1* RNA accumulation than that of cold acclimated RLD plants, the amount of *COR* transcript does not appear to be substantially greater (see Figure 2.2). There are several possible explanations for this observation. Firstly, the *CBF1*-transgene RNA may not be efficiently translated. To determine whether this is correct, CBF1 protein accumulation levels would need to be assayed in *CBF1*-overexpressing plants under both nonacclimating and acclimating

conditions and compared to control lines under the same conditions. However, despite repeated attempts, the CBF1 protein was not detected (See Chapter 3; K Jaglo, S. Gilmour and M. Thomashow, unpublished) making it impossible to definitively answer this question.

Secondly, the *CBF1* RNA may be efficiently translated into protein, but the protein does not bind and/or recruit cofactors as efficiently under nonacclimating conditions. Thirdly, under nonacclimating conditions, CBF1 may have a structural conformation such that it interacts with a repressor or some other molecule that sequesters it from binding to the CRT/DRE. It is now known that under acclimating conditions, the CBF1 protein denatures and elongates, a rare phenomenon for a monomeric protein under physiological conditions (Kanaya et al, 1999). This change in conformation may result in more efficient binding, or more efficient recruiting of co-factors under acclimating conditions. We have evidence that CBF1 is dependent on the ADA2, ADA3 and GCN5 cofactors for transcription activation in yeast (Stockinger et al, 1997; E. Stockinger, Y. Mao, S. Triezenberg and M. Thomashow, unpublished). If CBF1 is also dependent on these cofactors in Arabidopsis, then this dependence could support the second hypothesis that the increased abundance of *CBF1* RNA in the A6 line under nonacclimating and acclimating conditions does not result in a massive increase in *COR* RNA due to inefficient binding or inefficient recruiting of cofactors. Another possibility is that hypothesized by Kanaya et al (1999): the change in conformation under acclimating conditions may allow CBF1 to be released from a negative repressor. In this case, overexpression of the CBF1 protein would result in a positive change in the ratio between the CBF1 protein and repressor allowing for transcription activation under

nonacclimating conditions. Again it would be expected that activation would not be as efficient under nonacclimating conditions since some protein would still be inactive and bound to the repressor. However, until we can detect the CBF1 protein, we will not be able to determine which hypothesis, if any, is correct.

The final possibility is that the CBF1 protein may be modified under acclimating conditions. Many transcriptional activators are activated or deactivated by phosphorylation, methylation or the addition of other small molecules in response to the correct stimulus (Gallie, 1993; Schwechheimer et al, 1998). The CBF1 protein contains sites that are similar to mitogen activated protein (MAP) kinase phosphorylation sites (E. Stockinger and M. Thomashow, unpublished) suggesting that the protein may be phosphorylated under either acclimating conditions to activate the protein or under nonacclimating conditions to deactivate the protein. Given the high level of *CBF* RNA accumulation and relatively low level of *COR* RNA accumulation in A6 plants as compared to RLD plants, activation by phosphorylation seems more likely. Mitogen activated protein kinases are known to be activated at low temperatures in alfalfa (Jonak et al, 1996). Conceivably, homologues of these kinases could function to phosphorylate CBF1 in Arabidopsis and activate it under acclimating conditions. However, until the protein can be detected, whether or not CBF1 is modified by phosphorylation or some other small molecule will remain unknown.

As stated above, overexpression of *CBF1* results in increased *COR* gene expression under nonacclimating conditions. This correlative increase in both *CBF* and *COR* gene expression, along with the gel shift data indicating that CBF1 can bind to the CRT/DRE, and the data that CBF1 can activate a reporter gene under the control of the

COR15a promoter in yeast (Stockinger et al, 1998) are a strong indicator that *CBF1* is a regulator of *COR* gene expression. This is significant as it is the first transcription factor isolated that acts upstream of proteins known to be associated with cold acclimation, the *COR* proteins.

It is now known that *CBF1* is a member of a small gene family consisting of *CBF1, CBF2* and *CBF3* which are approximately 90% similar in amino acid sequence and all of which to bind to the CRT/DRE and activate transcription of a reporter gene in yeast (Gilmour et al, 1998). Overexpression of *CBF3* in Arabidopsis results in increased *COR* gene expression under both nonacclimating and acclimating conditions supporting the conclusion that all three CBF proteins are likely regulators of *COR* gene expression (Gilmour, 2000; S. Gilmour, M. Salazar and M. Thomashow, unpublished). Interestingly, *CBF3*-overexpressing plants have increased levels of proline and total soluble sugars compared to control lines, as well as increased transcript levels of *P5CS* which is involved in the production of free proline (Gilmour et al, 2000). *CBF3*-overexpressing plants also have changes in membrane lipids which are associated with some of the changes that occur during cold acclimation. Combined, these data indicate that CBF3 activates multiple pathways involved with cold acclimation and suggest that the *CBF* genes may play critical roles in inducing the many changes in gene expression associated with cold acclimation.

However, to determine definitively if all three CBF proteins are regulators of *COR* gene expression, all three *CBF* genes would need to be deleted and *COR* gene expression monitored. If deletion of all three *CBF* genes resulted in a loss of *COR* gene expression, this would be proof that the CBF proteins are the activators of *COR* gene

expression under acclimating conditions. By adding back each *CBF* individually, differences in activation between the CBF proteins could be investigated. However, it is possible that deletion of all three *CBF* genes may not have an effect on *COR* gene expression. This could either be an indication that the CBF proteins are not the natural regulators of *COR* genes, or that there is redundancy in *COR* gene activation. The second possibility would not be unexpected as the ABA induced activation of the *COR* genes is independent of cold and drought induced activation (Baker et al, 1994; Yamaguchi-Shinozaki and Shinozaki, 1994; Wang et al, 1995) indicating that several independent pathways may converge in the induction of the *COR* genes. The exact role of each *CBF* and differences in the genes they activate will remain unknown until triple *CBF* knockout lines are isolated.

While it has long been hypothesized that the *COR* genes play direct roles in increasing freezing tolerance, direct evidence has been lacking. Previous work has shown that overexpression of *COR15a* results in increased freezing tolerance of chloroplasts, but not of whole leaves (Artus et al, 1996, Figure 2.3B). The slight but significant increase in freezing tolerance brought about by overexpression of one COR protein raised the intriguing possibility that overexpression of the battery of COR proteins would increase freezing tolerance more dramatically. Here I present evidence that the *COR* genes play a role in increasing freezing tolerance since overexpression of the battery of *COR* genes results in increased freezing tolerance without a low temperature stimulus (see Figure 2.3, Figure 2.4, Figure 2.5A, Figure 2.6B, Figure 2.8A, and Table 2.1). These data are significant as they are strong evidence that overexpression of all the *CBF1*-induced *COR* genes results in increased freezing tolerance that can be observed at the level of whole

leaves, and for the A6 line, whole plants.

There appears to be a positive correlation between increased freezing tolerance and *COR* gene expression. The A6 line, which has higher levels of *COR* gene expression than the B16 line in the T4 generation (see Figure 2.2), also has a greater increase in freezing tolerance than the B16 line. As determined by EL_{50} values in electrolyte leakage assays, the A6 line has a ~3.2° C increase in freezing tolerance while the B16 line only has a ~1.3°C increase in freezing tolerance (see Table 2.1). Furthermore, the EL_{50} value of the A6 line was not significantly different from 7-10 day cold-acclimated RLD plants (see Table 2.1). Perhaps the A6 line was the only line to consistently survive whole plant freeze tests because it exhibits a similar increase in freezing tolerance to acclimated wild type plants (see Figure 2.4). When 5-10 day cold-acclimated RLD plants, nonacclimated *CBF1*-overexpressing A6, B16, K16 and *COR15a*-overexpressing T8 plants were frozen to -5° for varying amounts of time, only cold-acclimated RLD plants and plants in the A6 line consistently showed increased survival. The similarity in *COR* gene expression levels of the A6 line and cold-acclimated RLD plants and the ability of both types of plants to survive freezing are a good indication that COR protein accumulation is a critical factor involved in increasing freezing tolerance during cold acclimation.

Despite the increase in freezing tolerance as seen by electrolyte leakage assays, *CBF1*-overexpressing plants from the A6, and B16 lines in the T3 generation did not appear to have increased germination under osmotic stress. Due to the similarities between drought, cold and osmotic stress, (Thomashow, 1999) it was possible that *CBF1*-overexpressing plants might have increased tolerance to osmotic stress. I tested this hypothesis by doing germination assays on media containing differing levels of sodium

chloride. However, no differences were seen between the *CBF*-overexpressing lines and the control RLD plants as none of the plants germinated in sodium chloride levels of 150mM or higher. There are several possibilities as to why there were no differences seen between RLD plants and the *CBF1*-overexpressing lines. The experiment should be repeated with 5mM differences in salt concentrations from 125 – 150 mM to see if there are small differences between *CBF1*-overexpressing and control RLD plants in the ability to germinate on sodium chloride. Additionally, there are different genes involved between tolerance to germinate under osmotic stress and ability to grow in osmotically stressed conditions (Saleki, 1993). In our case, it could be that *CBF1*-overexpressing plants do not have an increased ability to germinate under osmotic stress, but have increased ability to grow under osmotic stress, as whether the *COR* genes are expressed in seeds is not known. To test this hypothesis, control and *CBF1*-overexpressing plants should be germinated under nonstressed conditions, then transferred to plates containing NaCl or mannitol, to test for differences in ability to grow under osmotic stress.

While overexpression of *CBF1* initially led to increased *COR* gene accumulation, several generations of self-pollination led to a reduction in *COR* gene expression in *CBF1*-overexpressing plants. The B16 plants in the T4 generation did not appear to have as dramatic an increase in freezing tolerance as B16 plants in the T3 generation (see Figure 2.5). This could be seen by both electrolyte leakage assays and RNA accumulation of *CBF1* and the *COR* genes. Similar patterns of reduced expression were noticed later with the A6 line in the T5 generation (see Figure 2.6) and the K16 line in the T3 generation (see Figure 2.8).

To determine what was causing the decrease in *COR* gene expression, individual

families of A6 T5 lines were isolated and checked for COR15m protein accumulation. Some of the lines had high levels of COR15m protein accumulation while others had little or no COR15m protein accumulation as compared to RLD plants under nonacclimating conditions (see Figure 2.6A). As the loss of COR15m protein accumulation could be an indication of either *CBF1* co-suppression, *CBF1* transgene silencing, or the physical loss of the *CBF1* transgene, experiments were conducted to determine which hypothesis was correct. Control RLD plants and the individual A6 T5 generation lines were cold-acclimated for seven days and COR15m protein accumulation was investigated (see Figure 2.6C). As COR15m protein accumulation did not appear to be significantly different between RLD plants and any of the individual families of the A6 T5 generation lines, I concluded that the endogenous copy of the *CBF1* gene was still functional and that co-suppression had not occurred (Figure 2.6C). However, the possibility that the *COR* gene expression detected was due to redundancy of activation by the other two *CBF* genes (of which I was not aware at the time) cannot currently be ruled out.

Electrolyte leakage assays on four selected A6 T5 lines indicated a positive correlation between *COR* gene expression and freezing tolerance (see Figure 2.6B). The lines with little or no COR15m protein accumulation (#15 and #9) had less of an increase in freezing tolerance as compared to lines with high levels of expression (#14 and #3). To determine if the amount of COR15m protein accumulation was identical in all the plants from each individual A6 T5 family, individual plants from the lines with low and high levels of COR15m protein accumulation were assayed for COR15m protein accumulation (see Figure 2.6D). Relatively consistent patterns of COR15m protein accumulation were

seen. In the two lines with high levels of expression, (#14 and #3), the majority of the six individual plants investigated had greater levels of COR15m protein accumulation than the control RLD plants under nonacclimating conditions. In the two lines with low levels of expression (#15 and #9), virtually no COR15m protein accumulation was detected. These data indicate that individual plants within A6 T5 families were experiencing loss of COR15m protein accumulation presumably as a result of loss of *CBF1*-transgene expression, although to know definitively, more individual plants from each family would need to be tested for COR15m accumulation. The families with high expression had fewer individuals with low expression (lines #14 and #3) whereas families with low levels of expression appeared to have all lost *CBF1*-transgene expression (lines #15 and #9).

To ensure that the loss of expression seen in some of the individual A6 T5 families was not due to loss of the transgene, Southern hybridization analysis was conducted (see Figure 2.6E). The *Hind* III –*CBF1* containing fragment was present in all of the A6 T5 families tested, and not in the RLD plants, regardless of the amount of COR15m protein accumulation. These data clearly indicate that the loss of expression was not due to the loss of the transgene, but was most likely due to transgene silencing.

Overexpression of a given gene in the antisense orientation can result in the loss of expression of the endogenous gene (van der Krol et al, 1990). Based on this knowledge, I transformed plants with antisense 35S:*CBF1*, pKJO2a (see section 2.2.2) in the hopes of reducing or eliminating *CBF1* gene expression. A total of 47 independent lines were investigated directly and indirectly for indications that endogenous *CBF1* gene expression had been reduced or lost. In the T2 generation, *CBF1* RNA accumulation

under nonacclimating conditions was assayed in the transgenic lines to determine if the transgene was being expressed (Figure 2.9A). As seen inFigure 2.9A, some of the antisense-*CBF1* lines have an increase in *CBF1* RNA accumulation indicating that the transgene was expressed. In the T3 generation, *COR15* RNA accumulation was investigated under both nonacclimating and acclimating conditions (Figure 2.9B), but no reduction in *COR15* RNA was seen in the antisense-*CBF1* line. Lastly, 32 lines were screened for COR15m protein accumulation under cold-acclimating conditions (Figure 2.9C). No dramatic differences were seen between the control and transgenic plants. It is important to note that to check for expression of antisense-*CBF1* RNA, total plant RNA was probed with a double stranded *CBF1* DNA probe, which hybridizes to both sense and antisense *CBF1* RNA. Given that no increase in *CBF1* RNA was seen in control plants, it was assumed that the increase in *CBF1* RNA accumulation was due to antisense *CBF1* RNA. The possibility that the increase in RNA could have been due to an increase in sense-*CBF1*, therefore, cannot be ruled out.

There are several possible reasons why a decrease in *COR* gene expression was not observed in the antisense lines under acclimating conditions. Firstly, *CBF1* is part of a small gene family consisting of three members (Gilmour et al, 1998). While the genes are 90% similar at the amino acid level, they are only approximately 70% identical in nucleic acid sequence which may be enough difference that the antisense-*CBF1* construct did not effect the expression of the endogenous *CBF2* and *CBF3* genes. It is entirely possible that the antisense plants did cause a reduction in *CBF1* RNA accumulation, but did not effect *CBF2* or *CBF3* RNA accumulation. With two other copies of unaffected *CBF* genes, a reduction in *COR* gene expression may not occur, as the expression of *CBF2* and *CBF3*

may have compensated for the reduction or loss of *CBF1* expression. Secondly, it is possible that the antisense-*CBF1* construct, pKJO2a, resulted in total loss of all three *CBF genes* which was lethal, thus making it impossible to isolate knockout plants. Thirdly, it is possible that *CBF1* knockout plants could be isolated using the described construct, but not enough lines were screened to isolate these lines. Lastly, it is possible that the antisense-*CBF1* may not have effected the expression of the endogenous *CBF1* gene. Waterhouse et al, (1998) have done elegant experiments indicating that overexpression of the antisense orientation of a gene does not always effect gene expression. However, expression of a short sequence of the sense orientation of the gene connected to the antisense orientation results in significant reductions of endogenous gene expression. It is possible that if such constructs were transformed into Arabidopsis plants, *CBF* gene expression could be reduced or eliminated. If so, studies to determine the *COR* gene expression and freezing tolerance of these lines could aid in our understanding of the roles of the *CBF* genes and the *COR* genes in cold-acclimation.

In summary, it has been determined that *CBF1* is an activator of the *COR* genes and is therefore likely to be one of the early activators involved in cold acclimation. While it has not been determined with absolute certainty that *CBF1* is one of the transcription factor(s) required for activation of the *COR* genes under acclimating conditions, overexpression of *CBF1* does result in increased *COR* gene expression without a low temperature stimulus. Additionally, overexpression of *CBF1* and the *COR* genes results in increased freezing tolerance as seen by electrolyte leakage assays and whole plant freeze tests. These data clearly indicate that *CBF1*-induced genes play a role in increasing freezing tolerance and give more direct evidence to the theory that the *COR*

genes are involved in increasing freezing tolerance. However, until the effects of blocking all *COR* gene expression are known, direct evidence of their specific roles will still be lacking.

The potential long term applications of the effects of *CBF1*-overexpression are exciting. Traditional plant breeders have long sought to increase freezing tolerance as, for example, the most freezing tolerant wheat varieties today are essentially identical to those from the turn of the century (Thomashow, 1999). If the CBF-induced signaling cascade is present in other plant species, and searches of GenBank (http: //www.ncbi.nim.nih.gov: 80/BLAST) show sequence similarities in numerous other plants species (see also Chapter 4), then manipulation of this family of transcription factors could be the key to increasing freezing tolerance in agronomically important species.

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3. CHAPTER 3: Detection of Arabidopsis CBF proteins by immunoblot analysis and immunoprecipitation

3.1 INTRODUCTION

There are many ways in which transcription factors can be regulated in a eukaryotic cell. These include transcriptionally, post-transcriptionally, translationally, post-translationally through modification, or combinations of the above (Gallie, 1993; Schwechheimer et al, 1998). Additionally, transcription factors can be regulated by their physical location in the cell. For example, some transcription factors are sequestered in the cytoplasm, then translocated to the nucleus when the correct stimulus is detected (Gallie, 1993; Schwechheimer et al, 1998).

Changes in gene expression are associated with cold acclimation, the process by which plants increase in freezing tolerance after exposure to low, nonfreezing temperatures, as was first demonstrated in spinach (Guy et al, 1985). Genes associated with cold acclimation, called COR (cold-regulated) -also called *LTI* (low temperature-induced), *KIN* (cold-inducible), *RD* (responsive to desiccation) and *ERD* (early dehydration-inducible), were later isolated in Arabidopsis. The *COR* genes, which include *COR6.6, COR15, COR47* and *COR78*, are greatly upregulated under cold-acclimating and drought conditions as well as by application of ABA (Hajela et al, 1990; Nordin et al, 1991; Wang et al, 1994; Welin et al, 1994).

A major breakthrough in understanding how *COR* genes are regulated by low temperature was the isolation of *CBF1, CBF2* and *CBF3* (CRT/DRE Binding Factor) also called *DREB1b, DREB1c* and *DREB1a* respectively (Dehydration Responsive Element Binding factor). The CBF/DREBs encode for a family of transcription factors which bind to the CRT/DRE (C-Repeat/Dehydration Responsive Element) present in *COR* gene promoters and activate transcription under cold-acclimating conditions (Stockinger et al, 1997; Gilmour et al, 1998; Liu et al, 1998; Shinwari et al, 1998). The three genes, *CBF1*, *CBF2* and *CBF3* are highly similar in the amino acid sequence (~85%), contain AP2-like DNA binding domains, putative acidic activation domains, and have been shown to activate transcription in yeast (Stockinger et al, 1997; Gilmour et al, 1998). Constitutive overexpression of *CBF1, CBF2* or *CBF3* in Arabidopsis results in constitutive *COR* gene expression and increased freezing tolerance under non-acclimating conditions (Jaglo-Ottosen et al, 1998; Liu et al, 1998; Gilmour et al, 2000; S. Gilmour, M. Salazar, A. Sebolt, and M. Thomashow, unpublished).

The three *CBF* genes are regulated at the level of RNA accumulation. RNA levels of all three *CBF* genes increase within 15 min of a low temperature stimulus, peak at two to four hours and remain at an elevated state for as long as the plants are under acclimating conditions (Gilmour et al, 1998). While these data indicate that the *CBF* genes are transcriptionally and/or post-transcriptionally regulated, CBF proteins may also be translationally regulated or post-translationally modified. Analysis of the amino acid sequence of the CBF2 protein shows that there are 17 predicted potential phosphorylation sites; 9 on serine residues, 6 on threonine residues and 2 on tyrosine residues (http: //www.cbs.dtu.dk/services/NetPhos/-; Blom et al, 1999). Additionally, one of the sites

resembles the recognition site for MAP kinases (E. Stockinger, M. Thomashow, unpublished). This raises the intriguing possibility that the CBF proteins could be post-translationally modified through phosphorylation.

Phosphorylation events can modify the activity transcription factor at three levels: import into the nucleus, enhancement or repression of DNA binding activity, and enhancement or repression of the activation potential (Hunter and Karin, 1992; Schwechheimer et al, 1998). There are data that are consistent with the possibility of a CBF-phosphorylation event under cold acclimating conditions. A low-temperature induced mitogen activated kinase was recently isolated from alfalfa (Jonak et al, 1996) which is activated within 10 min of exposure of plants to low temperatures. If a homologous kinase is present in Arabidopsis, then it is possible that the CBF proteins could be activated by a phosphorylation event under acclimating conditions. Alternatively, an upstream factor could be modified which would then activate the CBF proteins (Thomashow, 1999).

To date, the only data available on the regulation of the *CBF* genes are RNA accumulation data. To fully understand how the genes are regulated, information on the level to which the proteins are accumulated, and if the proteins are post-translationally modified is critical. I attempted to determine the amount of CBF protein accumulation in transgenic *CBF1- CBF2-* or *CBF3*-overexpressing plants and wild type Arabidopsis plants. This was done not only to determine the levels of CBF protein accumulation, but also to determine the location of the protein in the cell and whether the protein was post-translationally modified under nonacclimating or acclimating conditions. If a modification event was detected, the type of modification and whether the modification

worked to activate or repress the function of transcription activation of the *COR* genes would then be investigated. Additionally, determining the amount of protein accumulated and the location of the CBF proteins in transgenic plants as compared to wild-type nonacclimated and acclimated plants was of interest. The amount of *CBF1* RNA accumulated in the nonacclimated A6 line appears to be greater than that of four-day cold-acclimated control plants, whereas the amount of *COR* gene RNA that is accumulated appears equal in both types of plants. Determining the amount of CBF1 protein that is accumulated in both types of plants may give an indication as to why the *COR* gene RNA accumulation is not directly reflective of the amount of *CBF1* RNA accumulated.

3.2 MATERIALS AND METHODS:

3.2.1 PLANT MATERIAL

Types of plant material used in this chapter are as follows:

Plant material	Nomenclature
Wild type Arabidopsis plants:	ecotype RLD or WS
CBF1-overexpressing plants:	A6 (described in 2.2.2) G7a-1 *
CBF2-overexpressing plants:	E71-1 *
CBF3-overexpressing plants:	A30a-1 *

A38b-7 *

Vector control plants: B16-1 **

For the *CBF*-overexpressing lines, (*) the coding region of each specific *CBF* cDNA was cloned into the binary expression vector pGA643 (An, 1987) and plants were transformed by the floral dip procedure as described (Gilmour et al, 2000; M. Salazar, A. Sebolt, S. Gilmour M. Thomashow, unpublished). For the vector control plants (**) the pGA643 vector alone was transformed as described above.

3.2.2 PLANT GROWTH

3.2.2.1 Plant growth (no radiolabelling)

Arabidopsis plants were grown in pots under 100 μmol $m^{-2}s^{-1}$ continuous light for 18-25 days as described (Gilmour et al, 1988; see 2.2.1). For experiments involving cold-acclimation, plants were cold acclimated at 4° C under 50 μmol $m^{-2}s^{-1}$ continuous fluorescent illumination for various amounts of time as indicated (Gilmour et al, 1988; see 2.2.1).

3.2.2.2 Plant growth for ^{35}S-methionine radiolabelling

Arabidopsis plants were grown on petri plates or in magenta boxes containing 1 x Gamborgs B-5 media (GibcoBRL,Grand Island, NY) as recommended by the

manufacturer and solidified with 1% agarose. Plants were placed under 30-60 $\mu molm^{-2}s^{-1}$ florescent illumination in a 16/8 hr light/dark cycle. When petri plates were used, plants were grown in a cluster in the center of the plate and a total of ~15-50 19-day old plants were analyzed under both nonacclimating and acclimating conditions. When magenta boxes were used, 19-day old plants were thinned from ~15 to 4-8 individuals before adding the ^{35}S-methionine as described in 3.2.7.2. For experiments involving cold-acclimation, plants were put in the 4° C cold room as described in 3.3.3.1 for a total of 24 h

3.2.2.3 Plant growth for ^{32}P-orthophosphate radiolabelling

Arabidopsis plants were grown in magenta boxes containing 1 x Gamborgs B-5 media (GibcoBRL,Grand Island, NY) as recommended by the manufacturer solidified, with 1% agarose and grown under the light conditions described above (see 3.2.2.2). For use in experiments, 14-22 day old plants were thinned from ~15 to 4-8 individuals before the addition of ^{32}P-orthophosphate as described in 3.2.7.3. For the experiments involving cold-acclimation, plants were put in the 4° C cold room as described in 3.3.3.1 for a total of 6 h

3.2.3 ISOLATION OF RECOMBINANT CBF1 PEPTIDES FROM E. COLI *EXTRACTS AND YEAST*

3.2.3.1 Overexpression in E. coli

Full length *CBF1* (amino-acids 1-213), the N-terminal portion of *CBF1* (amino acids 1-115) and the C-terminal portion of *CBF1* (amino acids 116-213) were cloned into the pGEX expression vectors (Pharmacia Biotechnology) under the control of the *tac* promoter, which results in the production of GST tagged polypeptides (E. Stockinger, M. Thomashow, unpublished). The resulting plasmids, pEJS369, pEJS370 and pEJS371 respectively, were transformed into BL21 *E. coli* cells (E. Stockinger, M. Thomashow, unpublished). Translation of the recombinant CBF1 polypeptides was induced with isopropyl thiogalacto-pyranoside (IPTG) as recommended by the supplier (Pharmacia Biotechnology) after which cells were lysed using a French press (Spectronic Instruments, Rochester, NY). Total soluble proteins, which included 8 M urea solublized CBF1-containing inclusion bodies from the pellet, were incubated with glutathione-agarose beads (Sigma, St. Louis, MO) to purify the GST-labelled CBF1 polypeptides as described (Ausubel et al, 1987). In some cases, the GST tag was removed by cleaving with thrombin (Ausubel et al, 1987).

3.2.3.2 Overexpression in yeast

Yeast cells that overexpress CBF1 were created by placing the coding region of the *CBF1* cDNA into the pDB20.1 expression vector (kindly provided by S. Triezenberg) under the control of the yeast ADC1 promoter (Stockinger et al, 1997). Total protein extracts (kindly provided by E. Stockinger) were isolated following the methods of Rose and Botstein (1983). This involves growing cells to A_{600} = 0.8- 1.0, chilling them on ice, briefly centrifuging the cells, suspending the cells in breaking buffer (100 mM Tris-HCl

(pH 8), 1 mM dithiothreitol and 20% glycerol) adding 0.45- 0.5-mm glass beads (Sigma) vortexing the cells, then centrifuging for 15 s and isolating the supernatant.

3.2.4 SYNTHETIC PEPTIDE PRODUCTION AND MANIPULATION

In order to generate antibodies that specifically detect CBF1, the C-terminal sequence CWNHNYDGEGDGDV from the CBF1 protein was used as an epitope. This portion of the protein is divergent from the CBF2 and CBF3 proteins (Gilmour et al, 1998). To generate antibodies that were expected to detect all three CBF proteins, we selected an epitope, the N-terminal sequence, FSEMFGSDYEC, that is conserved in all three CBF proteins (Gilmour et al, 1998). A cysteine residue was added to the end of each peptide to link the peptide to a carrier protein. Peptides were synthesized at the Keck Foundation Biotechnology Resources Laboratory on a Protein Technologies Symphony Multiple Peptide Synthesizer at the request of the Biopolymers Facilities at the Howard Hughes Medical Institute (Harvard Medical School).

Short peptides and other molecules smaller than 3000- 5000 Daltons, are not large enough molecules to function as immunogens (Harlow and Lane, 1988). Therefore, to elicit an immune response, 2 mg of peptides were conjugated to maleimide-activated keyhole limpet hemocyanin (KLH) as described by the manufacturer (Pierce, Rockford, IL) and checked for efficient conjugation by using Ellman's reagent (Pierce, Rockford, IL). As an adjuvant to stimulate the immunoresponse, 192µl of TiterMax (prepared as recommended by the manufacturer) (CytRx Corporation, Norcross, GA), was mixed with

288 µl of the peptides conjugated to KLH (containing approximately 0.5-1 mg of peptide) immediately prior to injection. Two rabbits for each peptide (#58 and #62 for the N-terminal, #63 and #64 for the C-terminal), were injected with ~1 ml of the peptide-KLH/TiterMax mix by a professional ULAR employee in accordance with the ULAR guidelines (animal use form approval number 03/96-021-00). Rabbits were bled approximately every four weeks and boosted with KLH-peptide when the titer of the antisera dropped. Serum was obtained by centrifugation of the clotted blood to remove red blood cells.

3.2.5 ANTIBODIES USED

Three types of anti-CBF1 antisera raised against the three following epitopes along with anti-COR15a antibodies were used in this chapter,:

Epitope Used/Abbreviated name	Experimental details
Recombinant full length CBF1 (F1, F2)	Rabbit #4 and #5, bleed 110 days (E. Stockinger, unpublished)
Peptide of N-terminus of CBF1 (N1, N2)	Rabbit #58 and #62, bleed #4 unless otherwise indicated
Peptide of C-terminus of CBF1 (C1, C2)	Rabbit #63 and #64, bleeds #4 unless otherwise indicated
COR15a (COR15m)	Rabbit Larry (Artus et al, 1996)

Sera containing antibodies to full length CBF1 was raised to recombinant histidine-tagged CBF1 (Stockinger, et al, 1997; E. Stockinger, M. Thomashow, unpublished). Sera

to the CBF1 N- and C-terminal peptides were raised as described in 3.2.4. Sera to the COR15a protein was raised to recombinant COR15a polypeptides (Artus, 1996).

3.2.6 PURIFICATION OF ANTI-CBF1 SERA

3.2.6.1 Introduction

Sera containing anti-CBF1 antibodies from F1, F2, N1, N2, C1 and C2 (see 3.2.5) were purified in a variety of ways. These included IgG purification, purification against full length recombinant CBF1 (see section 3.2.3, expression vector pEJS369), purification against portions of CBF1 (see section 3.2.3, expression vectors pEJS370 and pEJS371) and purification against synthesized peptides (see section 3.2.4).

3.2.6.2 Purification of IgG

Protein A, a cell wall protein of *S. aureus*, contains four potential binding sites for antibodies (Harlow and Lane, 1988). Therefore, it is an ideal molecule with which to purify the IgG fraction from other proteins and molecules contained in crude rabbit sera. To purify the IgG portion of F1, F2, C1, C2, N1 and N2 (see 3.2.5), a standard protocol based on adsorption of the IgG fraction to Protein-A sepharose beads (Sigma) was followed (#18.12, Sambrook et al, 1989).

3.2.6.3 Immunoaffinity purification to recombinant CBF peptides

For purification of antibodies C1 and C2 (see 3.2.5) to full length CBF1 protein (see section 3.2.3, expression vector pEJS369) or the N- and C-terminal portions of CBF1 (see section 3.2.3, expression vectors pEJS370 and pEJS371 respectively), a standard protocol using recognition of the GST-tag (Pharmacia Biotechnology) was followed (Bar-Peled and Raikhel, 1996). Briefly, total *E. coli* protein lysates containing recombinant CBF peptides were isolated as described (3.2.3), then incubated with glutathione-agarose beads, (Sigma, St. Louis, MO) which bind the GST-labelled CBF1 polypeptides. The bound GST-CBF peptides are cross-linked to the glutathione-agarose beads and used to immunoaffinity purify anti-CBF antibodies from crude rabbit sera.

3.2.6.4 Immunoaffinity purification to synthetic peptides

To purify the anti-CBF1 antibodies contained in N1, N2, C1 and C2, raised against synthetic peptides (see 3.2.5), a standard protocol "purification of antibodies against your specific peptide using Sulfolink Gel" was followed according to the manufacturers instructions (Pierce, Rockford, Il). Conjugation was checked with Ellman's reagent as recommended by the manufacturer (Pierce, Rockford, Il). This protocol involves conjugating the synthetic peptides to a matrix, then using the bound peptides to immunoaffinity purify the antibodies of interest.

3.2.7 EXTRACTION OF PROTEINS FROM PLANTS

3.2.7.1 Extraction of unlabelled proteins

Plants were grown as described in 3.2.2.1. For immunoblot analysis, total soluble protein was extracted from by grinding frozen leaf tissue (about 150 mg) into 300-400µl of one of the following extraction buffers:

1) 3x loading buffer for tricine SDS- PAGE (see section 2.2.5; Schägger and von Jagow, 1987) (30% glycerol, 6% SDS and 3 x upper tricine buffer); or
2) "buffer A" containing 50 mM Tris-HCl (pH 8.0), 5% glycerol, 100 mM KCl, 1.5% (wt/vol) polyvinyl-pyrrolidone, and the following cocktail of protease inhibitors: 1mM PMSF, 1mM benzamide, 1mM behzamidine·HCl, 5 mM E-Amino-n-caprioic acid, 10 mM EGTA, 1µg/mg antipain, 1 µg/mg leupeptin 0.1 mg/ml pepstatin (Sigma) and 5 mM DTT.

Insoluble material was removed by centrifugation at 3,000 x g for 20 min at 4° C, and the remaining protein in the supernatant was quantitated by the Bradford dye-binding assay (Bio-Rad, Hercules, CA).

3.2.7.2 Extraction of ^{35}S-methionine labelled proteins

Plants were grown on media as described in 3.2.2.2. Labelling was conducted by adding 0.5 mCi of ^{35}S-methionine (44.5 µl) suspended in 155.5 µl 0.2% tween and

pipetting the 200 µl of solution onto the leaves of the plants, after which plates were wrapped with parafilm. For nonacclimating conditions, one set of each type of plant was placed in the radioactive-use hood under low light conditions (~15 $\mu molm^{-2}s^{-1}$) for 24 h at ~21° C. For cold-acclimating conditions, the second set of plants were placed at 4° C as described in 3.2.2.2 for 24 h. After the incubation, all plant material, including root material, was removed from plates using forceps. Plants were rinsed with deionized water and instantly frozen in liquid nitrogen in microcentrifuge tubes. Tissue was ground in 200 µl extraction buffer consisting of: 50 mM Tris-HCl pH 8.0, 100 mM KCl, 2 mM $MgCl_2$, 2 mM EDTA, 5% glycerol, 0.5% DOC, 5 mM DTT, ~1.5% (wt/vol) polyvinyl-pyrrolidone (Mao, unpublished) the Complete™ Mini EDTA-free protease inhibitor cocktail tablet (Boehringer-Mannheim GmbH, Mannheim, Germany) 1mM PMSF, 0.1 mg/ml pepstatin, 5 mM DTT and 5% B-mercapto-ethanol (Sigma). The insoluble material was removed by centrifugation at 13,000 x g for 20 min at 4° C. The supernatant was quantified for radioisotope incorporation using standard trichloroacetic acid (TCA) procedures (Sambrook et al, 1989) and stored at –80° C until use.

3.2.7.3 Extraction of ^{32}P-orthophosphate labelled proteins

Plants were grown on media as described in 3.2.3.3. Labelling was conducted by pipetting 0.5 mCi of ^{32}P-orthophosphate (100 µl) suspended in 200 µl 0.2% tween onto the leaves of plants, ~75 µl of radioactive material per plant, after which the magenta boxes were wrapped with parafilm. For nonacclimating conditions, one set of each type

of plant was placed in the radioactive-use hood under low light conditions (~15 μmolm^{-2}s^{-1}) for 6 h at ~21° C. For cold-acclimating conditions second set of plants were placed at 4° C as described in 3.2.2.2. After the 6 h incubation, all plant material, including root material, was then removed from plates using forceps and extracted as described in 3.2.7.2 with the modification that 15mM B-glycerophosphate and 5 mM NaF were added as phosphoinhibitors. The supernatant was quantified for radioisotope incorporation as described above (see 3.2.7.2) and stored at –80° C until use.

3.2.8 IMMUNOBLOT ANALYSIS

3.2.8.1 Analysis without immunoprecipitations

To analyze CBF protein content in whole plant extracts, immunoblot analyses were conducted as described in 2.2.5 with the following modifications. For recognition of the CBF1 protein, several forms of the three types of antibodies were used as indicated in the Figures. The specific anti-CBF1 antibody used and the level of purification are as follows:

Type of antibody	Level of purification
F1 and F2:	Unpurified IgG purified (see 3.2.6.2) Immunoaffinity purified to recombinant peptides (see 3.2.6.3)
C1, C2, F1, and F2	Unpurified IgG purified (see 3.2.6.2) Immunoaffinity purified to synthetic peptides (see 3.2.6.4)

Three types of secondary antibody, to detect the rabbit anti-CBF1 antibodies, were used as indicated in the Figures (Sigma, St. Louis, MO):

1) unpurified anti-rabbit immunoglobulin peroxidase conjugate; or

2) anti-rabbit IgG (whole molecule) peroxidase conjugate; or

3) monoclonal anti-rabbit immunoglobulins peroxidase conjugate.

Proteins were visualized using the ECL system (Amersham Buckinghamshire, UK).

3.2.8.2 Analysis after immunoprecipitations

For detection of protein accumulation after immunoprecipitations (see section 3.2.9) the protein-A agarose beads (to which the antibodies and protein of interest were bound) were washed, isolated and heated in 1 x extraction buffer (see sections 3.2.7.2 and 3.2.7.3) containing 5% SDS and 5% B-mercapto-ethanol (Laemmli, 1970). The supernatant was removed from the and loaded on 10% Laemmli gels (1970), transferred to nitrocellulose (see 2.2.5) and visualized by ECL (see 3.2.8.1).

3.2.9 IMMUNOPRECIPITATIONS OF CBF1 PROTEINS

Immunoprecipitations of nonradiolabelled plant extracts and ^{35}S-methionine labelled *E. coli* extracts were conducted using one of two following protocols: the modified Hondred (see 3.2.9.1) or the Mittlera (see 3.2.9.2).

3.2.9.1 Modified Hondred protocol

Following the modified Hondred protocol (Hondred et al, 1987, S. Gilmour, unpublished) plant extracts (see section 3.2.2.1, extraction buffer #2) or *E. coli* extracts (see section 3.2.10) were pre-incubated with rabbit serum-agarose beads and protein-A agarose beads (Sigma) for 1 h at 4° C with shaking to remove proteins that bind non-specifically to the beads. Specifically, 10 µl of each type of bead were added to 200µl of protein lysate. An equal volume of TNET/SDS (50mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2% Triton-X 100 and 0.2% SDS), the protease inhibitor cocktail (see section 3.2.2.1, extraction buffer #2), and an equal volume of tricine loading buffer (Schägger and von Jagow, 1987) and were added. To this solution, 10 µl of crude or IgG purified (described in 3.2.8.1) immune serum, or pre-immune serum was added. The solution was shaken for 15 min on ice after which 10 µl of protein-A agarose beads (Sigma) were added and the solution was shaken for an additional 15 min on ice. The supernatant was removed, the beads were washed three times with urea wash (2 M urea, 1% Triton-X 100, and 10 mM Tris·HCl pH 7.5), then one time with $T_{10}E_2$ (pH 7.5). Beads were heated to 100° C in 40 µl 1 x loading buffer with 5% B-mercapto-ethanol for 5 min and electrophoreased on a 10% tricine gel and visualized by immunoblot analysis (see section 3.2.8.2).

3.2.9.2 Mittlera et al protocol

Following the protocol of Mittlera et al (1998), tissue was extracted in ice cold 50 mM Tris·HCl (pH 8.0), 150 mM NaCl and 1% Triton-X 100 plus the cocktail of protease inhibitors (see section 3.2.2.1, protease inhibitors in extraction buffer #2). Three volumes of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA and 0.25% gelatin were added to the sample. The samples were centrifuged for five min at 10,000 x g at 4° C, the supernatant was quantitated by Bradford dye-binding assay (Bio-Rad, Hercules, CA) before use. Immunoprecipitation experiments were conducted by incubating protein extracts with 5 or 10µl of pre- or immune serum as described in the Figures, shaking the solution for three h on ice, after which 40 µl of protein-A beads were added and incubated for an additional 30 min on ice. The beads were washed three times with wash buffer (50 mM Tris-HCl (pH 7.5), 150mM NaCl, 0.1% Nonidet p-40 (NP-40) and 0.25% gelatin) resuspended in 40 µl 1 x loading buffer with 5% B-mercapto-ethanol, heated to 100° C in for 5 min and electrophoreased as described above (see section 3.2.9.1).

3.2.10 ISOLATION TOTAL SOLUBLE PROTEINS FROM ^{35}S-METHIONINE RADIOLABLLED E. COLI *FOR IMMUNOPRECIPITATIONS*

E. coli cells (BL21) that overexpress GST-CBF1 (see section 3.2.3, vector pEJS369) were grown and labelled as described (Harlow and Lane, 1988 p. 442, 458). Briefly, a single colony of pEJS369 overexpressing *E. coli* was inoculated into four ml of LB growth media and grown overnight with shaking. To obtain high-specific-activity

labelling, the cells were diluted 1/10 into M9 medium, (Harlow and Lane, 1988) and allowed to grow until an OD_{600} of 0.4 was reached, after which 0.1 mM IPTG and 100 μCi of ^{35}S-methionine were added. Cells were grown for another four h, placed on ice for 30 min after which cells were centrifuged for five min at 5,000 x g, washed with phosphate buffered saline (PBS) then resuspended in 10 cell volumes 50 mM glucose, 10 mM EDTA, 25 mM Tris·HCl (pH 8.0) 4 mg/ml lysozyme and the protease inhibitor cocktail (see section 3.2.2.1 buffer #2). Cells were incubated at ~21° C for five min, placed on ice, centrifuged for ten min at 10,000 x g at 4° C and resuspended in lysis buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS and 50 mM Tris·HCl, pH 8.0) and placed on ice for 30 min. The cells were then centrifuged for 10 min at 10,000 x g at 4° C after which the lysate was removed to a new tube. The pellet was re-suspended in 8 M urea and 0.1 M Tris·HCl (pH 8.0), centrifuged briefly and the supernatant added to the retained supernatant, while ensuring that the total urea concentration of the protein buffer remained <1 M. The proteins were aliquoted into microcentrifuge tubes and frozen at -20° C until use. Immunoprecipitations were conducted as described in 3.2.9.1.

3.2.11 IMMUNOPRECIPITATIONS WITH ^{35}S-METHIONINE LABELLED PROTEINS FROM ARABIDOPSIS PLANTS

For immunoprecipitations conducted with ^{35}S-methionine labelled proteins from Arabidopsis plants, plants were grown as described in 3.2.2.2. Plants were ^{35}S-methionine labelled, total soluble proteins were isolated, and the level of radioisotope incorporation was determined as described in 3.2.7.2. Protein extracts with equal counts of radioactivity

(2,940,000 counts) were used in all immunoprecipitation experiments.

Immunoprecipitation of CBF1 was conducted as follows. Protein extracts (isolated as described in 3.2.7.2) were pre-incubated over night on ice with 30 µl of each of protein-A agarose beads and rabbit serum-agarose beads (Sigma). The supernatant was removed, and 20 µl of pre- or immune serum from N2 (see section 3.2.5) was added and the solution was shaken on ice for 3 h after which 45 µl of protein-A beads (Sigma) were added and solution was shaken for another h. After completing the incubations, the solutions were centrifuged briefly, the supernatants removed and the beads were washed three times with 750 µl extraction buffer (see section 3.2.7.2) for two to five min with shaking. The beads were then resuspended in 40 µl 1 x Laemmli loading buffer (1970), heated to 100° C for five min in the presence of 5% B-mercapto-ethanol and loaded onto 10% Laemmli gels (1970). Gels were dried on a model 583 gel drier (BioRad) overnight and fluorography was performed with BioMax MS scientific imaging film and a BioMax MS intensifying screen (Kodak, Rochester, NY).

3.2.12 IMMUNOPRECIPITATIONS WITH ^{32}P-ORTHOPHOSPHATE LABELLED PROTEINS FROM ARABIDOPSIS PLANTS

For immunoprecipitations with ^{32}P-orthophosphate labelled proteins from Arabidopsis plants, plants were grown as described 3.2.2.3. Plants were ^{32}P-orthophosphate labelled, total soluble proteins were isolated, and the level of radioisotope

incorporation was determined as described in 3.2.7.3. Protein extracts with equal counts of radioactivity (140,000 counts) were used in all immunoprecipitation experiments. Protein extracts (see 3.2.2.3) were first pre-incubated overnight with 30 µl of protein-A agarose beads and 30 µl of rabbit serum-agarose beads (Sigma). The supernatant was removed and incubated with 20 µl of pre- or immune serum from C2 (see section 3.2.5) and 30 µl of protein-A beads for two nights. Beads were washed and loaded onto Laemmli gels, and dried as described in 3.2.11. Fluorography was performed with Hyperfilm MP (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, UK).

3.3 RESULTS:

3.3.1 RECOMBINANT CBF1 IS DEGRADED IN THE PRESENCE OF ARABIDOPSIS PROTEIN EXTRACTS

As CBF proteins had not been detected by immunoblot analysis in previous experiments (S. Gilmour, M. Salazar, and K. Jaglo-Ottosen, M. Thomashow, unpublished results), I first wanted to determine if the buffer used to extract soluble plant proteins was permitting the degradation of the CBF proteins. To answer this question, 100 ng recombinant CBF1 protein (see section 3.2.3) was added to leaf tissue from nonacclimated RLD plants and total soluble proteins were extracted (see 3.2.7.1). To determine if the specific buffer used and/or the addition of protease inhibitors had any effect on CBF degradation, two different types of buffer (see 3.2.7.1 #1 and #2) and

various protease inhibitors were used. Specifically, both buffers were used to extract proteins in the absence of protease inhibitors, or one of each of the following protease inhibitors were added to "buffer A" (see 3.2.7.1 #2) before extraction: 1μg/mg antipain, 1mM PMSF, 1 μg/mg leupeptin or 0.1 mg/ml pepstatinA (Sigma) after which immunoblot analysis was conducted using sera from F1 (data not shown). The recombinant CBF1 protein was degraded if no protease inhibitors were added to the extraction buffer resulting in the absence of a band where the CBF1 protein is predicted to run and all protease inhibitors appeared equally effective at blocking total degradation of CBF1. Therefore, either the described cocktail of protease inhibitors were added to all extraction buffers (see section 3.2.7.1 #2), or a Complete™ Mini EDTA-free protease inhibitor cocktail tablet was added to all extraction buffers as described by the manufacturer (Boehringer-Mannheim GmbH, Mannheim, Germany).

3.3.2 CBF PROTEINS ARE NOT DETECTED IN TOTAL SOLUBLE PLANT OR CBF1-OVEREXPRESSING YEAST PROTEIN EXTRACTS

Detecting CBF1 protein in total soluble protein extracts from leaf tissue of wild type or *CBF1*-overexpressing plants using crude rabbit sera proved difficult due to complex banding patterns (see Figure 3.1A). These are presumably due, in part, to the cross reactivity of proteins contained within crude anti-CBF1 rabbit sera to proteins present in total soluble plant extracts. IgG purification of rabbit serum used in immunoblot analysis can result in decreased background (Harlow and Lane, 1988). To determine if isolation of the IgG fraction of F2 (see section 3.2.5) decreased

"background" banding, the IgG fraction was purified from serum (see section 3.2.6.2). CBF1 protein accumulation was then determined with recombinant CBF1 protein, protein extracts from *CBF1*-expressing yeast, vector only-expressing yeast (Stockinger et al, 1997, see 3.2.3.2), nonacclimated RLD and A6 plants (see section 2.2.2, see 3.2.7.1 #2 for extraction buffer) and 5- and 8- day cold-acclimated RLD plants (see Figure 3.1). While 50 ng of recombinant CBF1 protein was detectable with both types of sera (the CBF1 protein is a doublet of ~ 29 KDa), CBF1 could not be detected in *CBF1*-overexpressing yeast or in plant extracts over "background" patterns. As CBF1 RNA accumulation increases within 15 min of touch stimulation (Gilmour et al, 1998) I wanted to determine if moving the plants from growth chambers to the lab before harvesting proteins had an effect on the ability to detect the CBF1 protein. However, the inability to detect CBF proteins was independent of where plant tissue was harvested as CBF proteins could not be detected regardless of whether plants were harvested in growth chambers or in the lab (data not shown).

3.3.3 PRE-IMMUNE SERUM CONTAINS ANTIBODIES TO NUMEROUS PROTEINS CONTAINED IN WHOLE PLANT EXTRACTS

To determine specifically what was causing the complex banding patterns seen in Figure 3.1, several control experiments were conducted. Immunoblot analysis was conducted using either pre-immune serum, no primary antibody or immune serum from F2 (see section 3.2.5) against 50 ng recombinant CBF1, total soluble proteins from nonacclimated RLD and *CBF1*-overexpressing A6 plants (see sections 2.2.2 and 3.2.7.1#2), and extracts from *CBF1*-overexpressing yeast cells (see 3.2.3.2) (Figure 3.2).

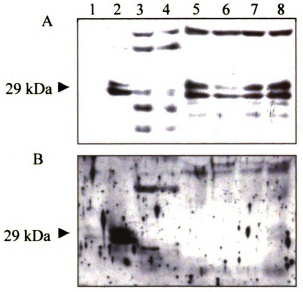

Figure 3.1. Immunoblot analysis of recombinant and native CBF1

Immunoblot analyses (see 3.2.8.1) were conducted using both IgG purified (1B, see 3.2.6.2) and crude F2 antisera (1A, see 3.2.5) against recombinant CBF1 protein (see 3.2.3.1) and soluble proteins from plant (see 3.2.1 for plant types and 3.2.7.1 #2 for protein extraction) and yeast extracts (see 3.2.3.2). In both immunoblots, the secondary antibody was unpurified (see 3.2.8.1 #1) used at a dilution of 1:5,000. The images are from a 5 minute exposure to x-ray film. The 29 kDa marker, the predicted size of CBF1, is indicated by the arrow.
Lanes are as follows:
1: 5 ng CBF1 recombinant protein;
2: 50 ng CBF1 recombinant protein
3: 100 µg total yeast protein extract (vector-only);
4: 100 µg total yeast protein extract (*CBF1*-expressing);
5: 100 µg protein extract from nonacclimated RLD plants;
6: 100 µg protein extract from 5-day cold-acclimated RLD plants;
7: 100 µg protein extract from 8-day cold-acclimated RLD plants;
8: 100 µg protein extract from nonacclimated A6 plants.

A. Immunoblot analysis using crude F2 antiserum diluted 1:50.
B. Immunoblot analysis using the IgG fraction purified from F2 antiserum diluted 1:500.

The immunoblot analyses indicated cross reactivity to yeast and plant proteins in the pre-immune serum of F2. Additionally, faint bands were seen when no primary antibody is used. This indicates that the secondary antibody alone has cross reactivity to plant proteins. Both of these factors are likely to increase background problems.

3.3.4 IMMUNOAFFINITY PURIFICATION OF ANTI-CBF1 ANTISERA DOES NOT ENHANCE DETECTION OF RECOMBINANT OR NATIVE CBF PROTEINS

Purification of the IgG portion of anti-CBF1 antiserum did result in a decrease in "background" banding, but did not allow for clear visualization of the CBF proteins (compare Figure 3.1A and B). Therefore sera from F1 and F2 (see section 3.2.5) were purified to full-length recombinant CBF1 peptides (see section 3.2.6.3) in an attempt to eliminate antibodies to non-CBF proteins contained within the antiserum. Immunoblot analysis using pre-immune serum, crude immune serum, and sera purified to recognize CBF peptides indicates that the purification process did not seem to effect recognition of the three CBF peptides (see Figure 3.3; data for F1 not shown). Given that serum purified to the C-terminal portion of CBF1 recognized the N-terminal portion of CBF1 (see Figure 3.3, "CBF1-C" section, lane 2), it is unclear that the purification process worked. However, given that there appear to be very few antibodies to the C-terminal portion of CBF1 in crude immune serum (see Figure 3.3, "Imm" section, lane 1), purification of these antibodies would not be possible. Additionally, there appear to be antibodies contained within the pre-immune serum that recognize the CBF1 protein. When immunoblot analyses were conducted against total soluble proteins from 4-day cold-

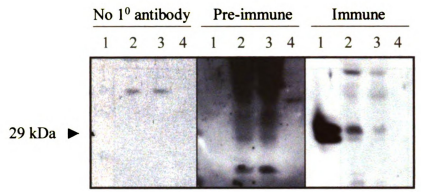

Figure 3.2. Immunoblot analysis of recombinant and native CBF1

Immunoblot analyses (see 3.2.8.1) were conducted using either no primary antibody (No 1^0 antibody), pre-immune serum (Pre-immune) or immune serum (Immune) from F2 (see 3.2.5) against recombinant CBF1 protein (see 3.2.3.1) and total soluble proteins from plant (see 3.2.1 for plant types and 3.2.7.1 #2 for protein extraction) and yeast extracts (see 3.2.3.2). Both pre- and immune sera were diluted 1:50. Images shown are the result of a 2 minute exposure to x-ray film for the pre- and immune sera and a 10 minute exposure for the No 1^0 antibody blot. In all blots, the secondary antibody was unpurified (see 3.2.8.1 #1) used at a dilution of 1:2,500. The 29 kDa marker, the predicted size of CBF proteins, is indicated by the arrow.
Lanes are as follows:
1: 50 ng CBF1 recombinant protein;
2: 100 μg protein extract from nonacclimated RLD plants;
3: 100 μg protein extract from nonacclimated A6 plants;
4: 25 μg protein extract from CBF1-overexpressing yeast.

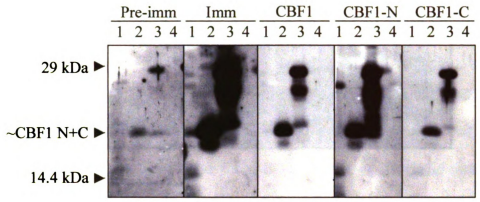

Figure 3.3. Immunoblot analysis of recombinant CBF1 peptides

Immunoblot analyses were conducted (see 3.2.8.1) with pre-immune (Pre-imm), crude immune (Imm), and recombinant full length (CBF1), N-terminal (CBF1-N), and C-terminal (CBF1-C) CBF1 peptide purified (see 3.2.6.3) serum from F2 (see 3.2.5).
The dilutions of the primary antibodies were as follows:
pre-immune: 1:1000;
crude immune: 1:1000;
CBF1 purified: 1:1000;
CBF1-N-terminal purified: 1:100;
CBF1-C-terminal purified 1:200.
In all blots, the secondary antibody was unpurified (see 3.2.8.1 #1) used at a dilution 1:5,000. All of the blots shown are the result of a 3 minute exposure except for the immune serum which is from a 30 second exposure. All recombinant protein extracts were thrombin cleaved. The 29 kDa, predicted size of CBF1, the approximate size of the CBF1-N and C terminal peptides and the the 14.4 kDa markers are indicated with arrows.
Lanes are as follows:
1: 1 μg C-terminal portion of recombinant CBF1;
2: 1 μg N-terminal portion of recombinant CBF1;
3: 1 μg full length recombinant CBF1;
4: 1 μg GST (Sigma)

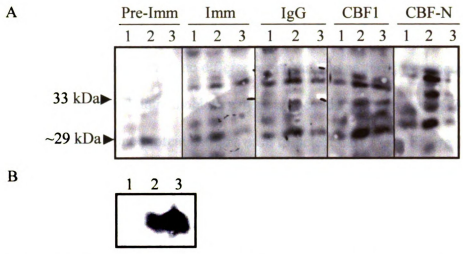

Figure 3.4. Immunoblot analysis of CBF and COR15m proteins

Lanes are as indicated:
1: Nonacclimated RLD
2: 4-day cold-acclimated RLD
3: Nonacclimated A6 (see 3.2.1)

A. Immunoblot analysis of CBF1. Immunoblot analyses (see 3.2.8.1) were conducting using pre-immune (Pre-Immune), crude immune (Imm), IgG purified (IgG) (see 3.2.6.2) and recombinant full length (CBF1), and N-terminal (CBF1-N) CBF1 peptide purified (see 3.2.6.3) serum from F2 (see 3.2.5). The dilutions of the primary antibodies are as follows:
Pre-immune: 1:5000;
Immune: 1:5000;
IgG: 1:3000;
CBF1: 1:1000;
CBF1-C-terminal purified 1:200;
CBF1-N-terminal purified: 1:300.
In all blots, the secondary antibody was unpurified (see 3.2.8.1 #1) used at a dilution 1:5,000. The 33 kDa marker, and the approximate position of 29 kDa, predicted size of CBF1, are indicated with arrows.
B. Immunoblot analysis of COR15. Immunoblot analysis was conducted as described (3.2.8.1) using antiserum raised to COR15 (see 3.2.5) diluted 1:2000. The secondary antibody was unpurified (see 3.2.8.1 #1) used at a dilution 1:5,000.
All of the blots shown are the result of a 30 second exposure to x-ray film.

acclimated RLD plants and nonacclimated RLD and A6 plants and using pre-immune serum, crude immune sera, IgG purified serum(see 3.2.6.2) and recombinant CBF1-peptide purified sera (see 3.2.6.3) no differences in banding patterns were detected (see Figure 3.4A). As a control, immunoblot analysis was conducted using anti-COR15a antibodies against the same protein extracts used to detect CBF1 (see Figure 3.4B). The data clearly show accumulation of the abundant COR15m protein. This indicates that the inability to detect CBF proteins is not due to technical difficulties in conducting the immunoblot analysis. Collectively, these results indicate that the purification process was ineffective at allowing for detection of the endogenous plant CBF proteins, possibly due to the fact that purification process was unsuccessful.

3.3.5 ANTIBODY PRODUCTION TO SPECIFIC PEPTIDES FROM THE CBF1 PROTEIN

Despite the repeated efforts in using sera F1 and F2 (see section 3.2.5), I was unable to detect CBF proteins in whole leaf plant extracts (Jaglo-Ottosen et al, 1998; Figures 1 and 2). This could be due to low levels of CBF1 protein accumulation, the anti-CBF1 antibodies having a weak affinity for the CBF1 protein, or a combination of both. However, given that 50 ng of recombinant CBF1 protein was detected, it seems more likely that CBF proteins do not accumulate to high levels in plants. Using antibodies against full length CBF1 also caused concerns. Depending on which part of the protein formed the epitope for the immunoresponse, it was possible that other AP2-domain containing proteins could be detected by anti-CBF1 antibodies. Additionally, since a large portion of the amino acid sequence is highly conserved between CBF1, CBF2 and

CBF3, it would be impossible to distinguish between the three proteins with anti-CBF1 antibodies. To avoid these problems, I decided to make synthetic peptides of small portions of the CBF1 protein to use as epitopes for raising new anti-CBF antibodies in rabbits. All portions of the CBF1 protein were initially checked for hydrophilicity (Kyte-Doolittle), surface probability (Emini) and antigenicity (Jameson-Wolf), using Protean in DNAStar (Madison, WI) to determine which portion of the protein would be best for the creation of peptides (Grant, 1972). To be able to detect the CBF1 protein specifically, a C-terminal sequence which is divergent between the three CBF proteins (Gilmour et al, 1998) and should only detect CBF1 protein was used. To generate antibodies to detect all three CBF proteins, an N-terminal epitope, that is conserved in all three CBF proteins (Gilmour et al, 1998) was selected. Antibodies were made as described in 3.2.4.

3.3.6 ALL TESTED PRE-IMMUNE SERA FROM RABBITS HAVE ANTIBODIES TO NON-SPECIFIC PLANT PROTEINS

In order to avoid high levels of cross reactivity to non-CBF plant proteins in the pre-immune serum, the pre-immune sera from prospective anti-CBF1 antibody producing rabbits were screened by immunoblot analysis. Of six rabbits tested, all had antibodies to plant proteins present in total soluble protein extracts from nonacclimated and acclimated leaves (data not shown). However, as the banding pattern indicated that there were no distinctive bands at ~29 kDa, the predicted size of the CBF proteins, the four rabbits that gave pre-immune serum that was least reactive to total soluble leaf extracts were selected. For the N-terminal peptides #58 and #62 were selected, for the C-terminal peptides, #63

and #64 were selected (see sections 3.2.4, and 3.2.5).

3.3.7 ANTISERA RAISED TO SYNTHETIC CBF PEPTIDES RECOGNIZE RECOMBINANT CBF1 PROTEIN, BUT CBF PROTEINS ARE NOT DETECTED IN PLANT EXTRACTS

All bleeds were titered to determine which dilutions gave maximum visualization of CBF proteins and minimum background patterns (data not shown). Despite the fact that immune serum from all four rabbits contained anti-CBF1 antibodies that could detect recombinant CBF1 protein, immunoblot analysis with total soluble plant extracts gave complex "background" banding patterns as seen with F1 and F2 (see 3.2.5) (see Figure 3.5A, C1 and N1). The complex "background" banding patterns resulted in the inability to definitively detect CBF proteins in total soluble leaf extracts from cold-acclimated RLD and nonacclimated RLD and A6 (see section 2.2.2) plants.

3.3.8 IMMUNOAFFINITY PURIFICATION OF ANTI- PEPTIDE ANTIBODIES REDUCES BACKGROUND, BUT CBF PROTEINS ARE NOT DETECTED IN PLANT EXTRACTS

As the crude sera from anti-CBF peptide antibodies resulted in complex banding patterns in immunoblot analyses using plant extracts, antibodies were immunoaffinity purified to synthetic peptides (see section 3.2.6.4). No differences were seen between purified and crude sera in immunoblot analyses using total soluble plant extracts, raising the possibility that the purification process was unsuccessful (see Figure 3.5A). There are many possible reasons for the complex banding pattern. One possibility was that it was

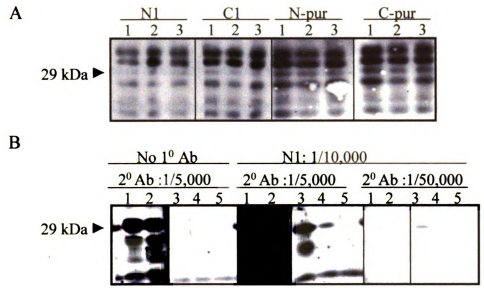

Figure 3.5. Immunoblot analysis of recombinant and native CBF1

A. Immunoblot analysis with crude and purified antisera against total soluble plant extracts. Immunoblot analyses were conducted as described (3.2.8.1) using crude N1 (N1) or C1 (C1) (see 3.2.5) and synthetic peptide purified (see 3.2.6.4) N1 and N2 combined (N-pur) or C1 and C2 combined (C-pur). All primary sera were diluted 1:100,000, the secondary antibody was IgG purified (see 3.2.8.1 #2) diluted 1:5,000. Lanes are as follows:
1: 3-day cold-acclimated A6 (see 3.2.1);
2: 3-day cold-acclimated RLD (see 3.2.1);
3: nonacclimated RLD (see 3.2.1).
The 29 kDa marker, the predicted size of the CBF proteins, is indicated with an arrow.

B. Immunoblot analysis of recombinant and native CBF1 protein. Immunoblot analyses were conducted as described (3.2.8.1) using no primary antibody (No 1^0 Ab) or N1 (see 3.2.5) diluted as described. The secondary antibody was IgG purified (see 3.2.8.1 #2) and diluted as indicated. Lanes are as follows:
1: 100 µg nonacclimated RLD; 2: 100 µg 3-day cold-acclimated RLD;
3: 300 ng CBF1; 4: 30 ng CBF1;
5: 3 ng CBF1.
The 29 kDa marker, the predicted size of the CBF proteins, is indicated with an arrow.

again due to cross-reactivity of the secondary antibody with proteins in total soluble protein extracts from leaf tissue. To test this hypothesis, immunoblot analyses were conducted in the absence of a primary antibody (see Figure 3.5B, "no 1° antibody" section lanes 1 and 2). There is an interaction between the secondary antibody and nonspecific proteins in total soluble leaf extracts. This interaction was seen regardless of whether unpurified goat anti-rabbit antibodies, IgG purified goat anti-rabbit antibodies, or monoclonal goat anti-rabbit antibodies (see section 3.2.8.1) were used in immunoblot analyses (data not shown).

One way to reduce or eliminate the interaction of the secondary antibody with nonspecific plant proteins is to change the initial blocking buffer used. Therefore, I experimented with different blocking agents to see if any of them resulted in a reduction in background banding. Bovine Serum Albumin, (BSA) (5%) resulted in less background than 5% non-fat powdered milk, or 10% rabbit serum, therefore 5% BSA was used in all the following immunoblot analyses (data not shown).

Another potential way to reduce complex banding patterns was to remove the non-specific plant proteins with which the secondary antibody interacts. To do this, I conducted immunoblot analyses using total soluble proteins isolated from roots, protoplasts (kindly donated by Yaopan Mao) and from nuclei (kindly donated by Charlie Herman). The background banding patterns with the three alternative protein sources were different than those seen in total soluble leaf extracts, but CBF proteins could still not definitively be detected (data not shown). Additionally, in the case of the protoplast extracts, the same complex banding patterns were visible with the pre- and immune serum, indicating that nonspecific interactions were still occurring.

Using the maximum possible dilution of the primary and secondary antibodies is another way to reduce non-specific banding patterns. Therefore, I wanted to determine the maximum possible dilution of antibodies where recombinant CBF1 protein could still be visualized. Additionally, I wanted to determine if nonspecific interactions occurred in immunoblot analyses with total soluble plant extracts using identical dilutions (Figure 3.5B). All three amounts of recombinant CBF1 protein, 300, 30 and 3 ng, (lanes 3-5) can clearly be seen in the 2° Ab: 1/5,000 blot. However, under identical conditions, immunoblot analysis using total soluble plant extracts resulted in very high levels of background banding patterns (lanes 1 and 2). This level of nonspecific binding would undoubtedly mask detection of CBF proteins. When the secondary antibody is used at a dilution of 1: 50,000 (2° Ab: 1/50,000) there are no background banding patterns seen in immunoblot analyses against total soluble plant proteins (lanes 1 and 2). However, at this dilution, 300 ng of CBF recombinant protein, an amount which is predicted to be several fold greater than that found in 100 μg total soluble leaf extracts, is only just detectable (lanes 3-5). Therefore, these data indicate that CBF proteins in plant extracts probably cannot be detected with the present antibodies using immunoblot analysis against total soluble plant proteins due to nonspecific banding patterns which mask visualization of the CBF proteins.

3.3.9 RECOMBINANT CBF1 MAY BE DETECTED BY IMMUNOPRECIPITATION, BUT NOT IN THE PRESENCE OF PLANT EXTRACTS

Detection of the CBF proteins by immunoblot analysis proved to be problematic

(see Figure 3.5B). Therefore, I decided to try detecting the CBF proteins by immunoprecipitation. By enriching for the CBF proteins and reducing the amount of other plant proteins, it was anticipated that the CBF proteins could be detected. For the first experiment, I wanted to determine if the CBF1 recombinant protein could be immunoprecipitated using N1, N2, C1 and C2 (see section 3.2.5). Previous work had indicated that the CBF proteins could adhere to microcentrifuge tubes and cause background problems (S. Gilmour, E. Stockinger, M. Thomashow, unpublished). Therefore, non-stick surface microcentrifuge tubes were used for all immunoprecipitation experiments (VWR Scientific Products, W. Chester, PA). Figure 3.6A shows the results of an immunoprecipitation experiment performed using 300 ng recombinant CBF1 protein as described (see section 3.2.9.1) with the exception that the proteins were not pre-cleared with rabbit-serum agarose beads (Sigma) before the experiment was conducted. The CBF1 protein is not immunoprecipitated without the addition of protein-A linked agarose beads (lanes 2-4) (Sigma) indicating that the CBF1 protein is not adhering to the microcentrifuge tubes. Additionally, the CBF1 protein is not immunoprecipitated if water instead of serum is added (lane 5). The pre-immune sera from N1 (lane 6), N2 (lane 9), C1 (lane 11) precipitated some of the CBF1 recombinant protein, but immune sera from N2 (lane 10) and C2 (lane 14) gave the best recovery of the recombinant protein. IgG purification of C1 (lane 8)(see section 3.2.6.2) did not seem to have any effect on the amount of CBF1 protein immunoprecipitated (compare to lane 7, unpurified C1). While the sample containing 300 ng recombinant protein loaded on a tricine gel (Schägger and von Jagow, 1987) appears as a doublet (see Figure 3.6, lane 1), all of the immunoprecipitated proteins appear to result in one band of a lower molecular

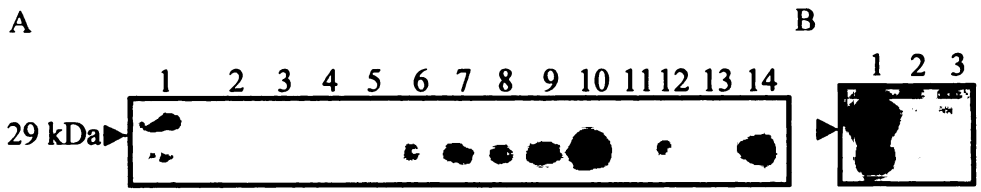

Figure 3.6. Immunoprecipitation of recombinant CBF1 protein followed by immunoblot analysis

A. Immunoprecipitation of recombinant CBF1 protein.
Immunoprecipitations were conducted as described (see 3.2.9.1), with the exception that protein extracts were not precleared, using N1, IgG purified N1 (3.2.6.2) N2, C1, and C2 (see 3.2.5). 300 ng of recombinant CBF1 protein (see 3.2.3) 10 µl pre- or immune sera and 10 µl of protein-A beads were used in each experiment unless otherwise indicated. Immunoblot analyses were conducted as described (3.2.8.1) using N1 (see 3.2.5) diluted 1/5,000. The secondary antibody was IgG purified (see 3.2.8.1 #2) used at a dilution of 1/100,000. The 29 kDa size marker, the predicted size of CBF1, is indicated with an arrow. Lanes are as follows:

1: 300 ng recombinant CBF1 protein;	2: water (no serum), no beads;
3: N1 pre-immune serum, no beads;	4: N1, no beads;
5: water (no serum);	6: N1 pre-immune serum;
7: N1;	8: N1 IGG purified
9: N2 pre-immune serum;	10: N2;
11: C1 pre-immune serum;	12: C1;
13: C2 pre-immune serum;	14: C2.

B. Immunoprecipitation of recombinant CBF1 protein in the presence of 7-day cold-acclimated RLD plant extracts. Immunoprecipitations were conducted as described (3.2.9.2) using 10 µl N1 (see 3.2.5) and 10 µl of protein-A beads. The immunoblot analysis was conducted with C2 diluted 1/1,000, the secondary antibody was IgG purified (see 3.2.8.1 #2) diluted 1/30,000. The 29 kDa size marker, the predicted size of CBF1, is indicated with an arrow. Lanes are as follows:
1: 3 µg recombinant CBF1;
2: 3 µg CBF1 incubated with 3 mg 7-day cold acclimated protein extracts;
3: 3 mg 7-day cold acclimated RLD protein extract.

weight. The reason for this difference is not known.

Recombinant CBF1 protein is degraded when added to total soluble plant extracts in the absence of protease inhibitors. Therefore, I wanted to determine if recombinant CBF1 could be immunoprecipitated in the presence of total soluble plant extracts with added protease inhibitors (see Figure 3.6B, and section 3.2.7.1 #2 for protease inhibitors and section 3.2.9.2 for the immunoprecipitation protocol). As a control, 3 μg CBF1 recombinant protein can clearly be detected (see lane 1). However, no CBF1 protein can be detected after immunoprecipitation of 3 μg CBF1 recombinant protein in the presence of 3 mg total soluble plant extracts from 7-day cold acclimated RLD plants using C1 (see lane 2). Additionally, no CBF proteins were detected after immunoprecipitation using 3 mg total soluble plant extracts from 7-day cold acclimated RLD plants (see lane 3).

3.3.10 CBF PROTEINS ARE NOT DETECTED BY IMMUNOPRECIPITATION FROM PLANT EXTRACTS

As immunoprecipitation of recombinant CBF1 in the absence of total soluble plant extracts appeared successful (see Figure 3.6A) I attempted to immunoprecipitate native CBF proteins from total soluble plant extracts as described (section 3.2.9.1, see Figure 3.7A). In this case, all protein extracts were pre-cleared with rabbit serum agarose (Sigma) to reduce non-specific binding. Surprisingly, in the lanes resulting from pre-clearing the total soluble plant proteins with rabbit-serum agarose beads (Sigma) ~29 kDa bands, the predicted size of the CBF proteins, appear to be present (see lanes 2-4. The white area is due to air bubbles). However, no distinct bands are present in any of the lanes where immunoprecipitations were conducted against 4-day cold acclimated RLD

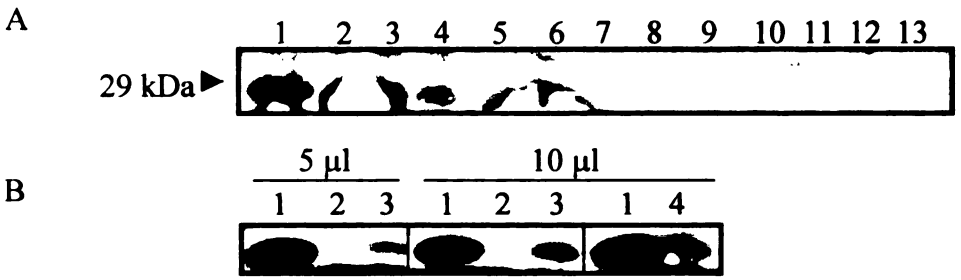

Figure 3.7. Immunoprecipitation of CBF and COR15 proteins from plant extracts.

A. Immunoprecipitation of CBF proteins from total plant extracts. Immunoprecipitations were conducted as described (see 3.2.9.1) using 10 µl of pre- or immune sera from N2 or C2 (see 3.2.5) as indicated and 10 µl of protein-A beads against 2 mg total soluble plant extracts. Plant types were nonacclimated (NA) and four-day cold acclimated RLD and A6 (CBF1-overexpressing) plants (see 3.2.1). Immunoblot analyses were conducted as described (see 3.2.8.1) using N1 diluted at 1/5,000 and IgG purified secondary antibody (see 3.2.8.1 #2) diluted 1;100,000. The 29 kDa size marker, the predicted size of CBF1, is indicated with an arrow. The lanes are as follows:

1: 300 ng recombinant CBF1 protein; 2: rabbit-serum agarose beads (RSA), NA RLD;
3: RSA, 4-day CA RLD 4: RSA beads, CA A6;
5: water (no serum), NA RLD; 6: C2 pre-immune, NA RLD;
7: C2, NA RLD; 8: C2, pre-immune, CA RLD;
9: C2, CA RLD; 10: water (no serum), CA A6;
11: C2 pre-immune, CA A6; 12: C2, CA A6 extracts;
13: Ca bleed #4, CA A6 extracts.

B. Immunoprecipitation of recombinant COR15. Immunoprecipitations were conducted as described (see 3.2.9.2) using 5 or 10 µl pre- or immune serum (see 3.2.5) as indicated and 10 µl protein-A beads against 100 ng recombinant COR15a. Immunoblot analyses were conducted with COR15 diluted 1/10,000, secondary antibodies were IgG purified (see 3.2.8.1 #2) and diluted 1/10,000. The lanes are as follows:

1: 100 ng COR15am; 2: COR15 pre-immune;
3: COR15 immune; 4:COR 15, 100 ng COR15am inl mg 7-day CA RLD.

(lane 9) and A6 plants (lane 12) using serum from C2 (see 3.2.5). One immunoprecipitation was conducted using serum from N2 (see 3.2.5) against 4-day cold acclimated A6 plants (lane 13). No distinct bands are observed at ~29 kDa, the predicted size of the CBF proteins. The experiment was repeated several times in order to investigate why the CBF proteins were not seen. Changing the volume in which the immunoprecipitation was conducted (from 100-400 µl) or the type (C1, C2, N1 or N2 (see 3.2.5), unpurified or purified (see 3.2.6.2 and 3.2.6.4) or amount (diluted 1/2,000 to 1/30,000) of primary antibody or the type (crude, IgG purified, and monoclonal, see 3.2.8.1) or amount (diluted 1/5,000 to 1/100,000) of secondary antibody used in immunoblot analyses did not result in the ability to detect native CBF proteins (data not shown). To check if the failure to immunoprecipitate CBF1 was due to technical difficulties, COR15m was immunoprecipitated (see Figure 3.7B, section 3.2.9.2). Lane 1 under all headings is 100ng COR15a protein loaded directly onto the gel as a positive control. Immunoprecipitation of recombinant and native COR15m is clearly possible under the given conditions (see lanes 3 and 4 under both headings), indicating that it is not due to technical difficulties that CBF proteins are not detected. Additionally, it appears as though 10 µl of anti-COR15a serum results in a better recovery of COR15a than using 5 µl (see "10µl" lanes 3 and 4 as opposed to "5µl" lane 3). However, it is important to note that the recovery percentage of the COR15a protein in both cases is low ~10-20%. If CBF proteins are recovered at an equally low percentage level, recovery amounts may be below the limits of protein detection using immunoblot analysis.

3.3.11 ^{35}S-METHIONINE LABELLED RECOMBINANT CBF1 CAN BE DETECTED BY

IMMUNOPRECIPITATION

To increase the level of detection of the CBF proteins, I attempted to radiolabel the proteins with ^{35}S-methionine. This eliminates the need to conduct immunoblot analysis and was expected to allow the detection very low levels of protein, as few as 10^3- 10^5 molecules per cell (Harlow and Lane, 1988). Immunoprecipitations were conducted with proteins isolated from *CBF1*-overexpressing *E. coli* (section 3.2.3, construct pEJS369) grown in the presence of 100 μCi of ^{35}S (section 3.2.10) (see Figure 3.8). Antisera from N2, C2 and F1 (see section 3.2.5) appeared able to immunoprecipitate *E. coli*-expressed CBF1 (Figure 3.8, see lanes 6, 8 and 10 under the "CBF1" heading) . However, as was seen before, it appears that the pre-immune sera from N2, C2 and F1 also immunoprecipitate CBF1 (Figure 3.8, see lanes 5, 7 and 9 under the "CBF1" heading), with F1 pre-immune resulting in almost identical amounts of protein to that seen in immune sera (Figure 3.8, compare lanes 9 and 10 under the "CBF1" heading). Alternatively, the increase in abundance of the putative CBF protein after immunoprecipitation could be due to nonspecific binding of the protein to the protein-A beads (Sigma).

The putative CBF1 protein immunoprecipitated by F1, N2 and C2 has a significantly higher molecular weight than the predicted weight of CBF1, ~29 kDa. This is presumably due to the presence of a 27.5 kDa GST tag (section 3.2.3, construct pEJS369). To ensure that the immunoprecipitated band was recombinant GST-tagged CBF1, total *E. coli* protein extracts were cleaved with thrombin to remove the GST tag (see Figure 3.8, lane 3 vs. 1). In the thrombin-cleaved GST-CBF1 extracts (Figure 3.7,

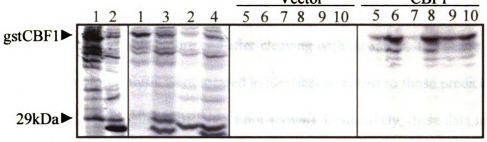

Figure 3.8. Immunoprecipitation of recombinant ^{35}S-Methionine labelled CBF1 from total *E. coli* protein extracts.

Proteins were isolated from *E. coli* radiolabelled with ^{35}S-methionine (see 3.2.10). Immunoprecipitations were conducted (see 3.2.9.1) using 20 µl *E. coli* protein extract transformed with vector (Vector) or *CBF1* (CBF1) and 20 µl pre- or immune serum from F1, N2 or C2 (see 3.2.5) as indicated and and 30 µl protein-A beads. The GST-tagged CBF1 position and the 29 kDa marker are indicated with arrows. The band in lane 2 is the 27.5 kDa GST protein. Lanes are as indicated:
1: 2 µl extract from *E. coli* transformed with *CBF1*;
2: 2 µl extract from *E. coli* transformed with vector;
3: 5 µl thrombin cleaved extract from *E. coli* transformed with *CBF1*;
4: 5 µl thrombin cleaved extract from *E. coli* transformed with vector;
5: N2 pre-immune;
6: N2;
7: C2 pre-immune;
8: C2;
9: F1 pre-immune;
10: F1.

The images for the two panels on the left are from a 9 day exposure to x-ray film at room temperature, images for the two panels on the right are from an over night exposure at room temperature.

lane 3) two bands of ~27 and 29 kDa are present, as would be predicted for the GST and CBF1 proteins respectively. Additionally, immunoblot analyses were conducted on GST-CBF1 protein extracts before and after cleaving with thrombin. The bands detected by immunoblot analysis were located in identical positions to those predicted to be the two forms of ^{35}S -labelled CBF1 (data not shown). Collectively, these data indicate that sera from F1, N2 and C2 can immunoprecipitate ^{35}S-methionine labelled recombinant CBF1 protein from total *E. coli* protein lysates.

3.3.12 ^{35}S-METHIONINE LABELLED RECOMBINANT CBF1 IS DEGRADED IN THE PRESENCE OF PLANT EXTRACTS

The ultimate goal of immunoprecipitation experiments with anti-CBF antibodies was to detect the native CBF proteins in wild type and *CBF*-overexpressing plants. Recombinant CBF1 protein appeared to be immunoprecipitated from total *E. coli* lysates (see Figure 3.8, see lanes 6, 8 and 10 under the "CBF1" heading). Therefore, the next step was to determine if the recombinant ^{35}S -labelled CBF1 protein could be immunoprecipitated in a solution containing plant proteins. Specifically, I wanted to determine if ^{35}S -labelled recombinant CBF1 was degraded in the presence of plant extracts as an indication that native CBF proteins would also be degraded. To test this, Arabidopsis plants were grown on media (see section 3.2.2.2 with the exception that plants themselves were not radiolabelled) and total soluble proteins were extracted from non-acclimated RLD and A6 plants as described (section 3.2.9.2). A total of 5 µl of ^{35}S –

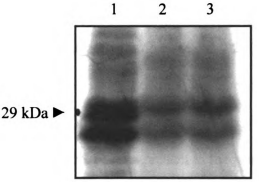

Figure 3.9. ^{35}S-methionine labelled recombinant CBF1 is degraded in the presence of plants and protoplast extracts.

Incubation of recombinant ^{35}S-methionine labelled CBF1 in plant extracts. Proteins were isolated from CBF1-overexpressing *E. coli* radiolabelled with ^{35}S-methionine, thrombin cleaved and incubated with plant extracts (see 3.2.7.1 #2) or protoplast extracts (see 3.2.7.2 for buffer). A total of 5 µl of *E. coli* extracts were incubated with plant extracts, protoplast extracts or water on ice for a total of 3 h. The 29 kDa size marker, the predicted size of CBF1, is indicated with an arrow. The lanes are as indicated:

1: 5 µl extract from *E. coli* transformed with *CBF1* in 30 µl water;
2: 5 µl extract from *E. coli* transformed with *CBF1* in 25 µl plant extracts;
3: 5 µl extract from *E. coli* transformed with *CBF1* in 20 µl protoplast extracts.

The image is from an 11 day exposure to x-ray film at room temperature.

labelled, thrombin cleaved *E. coli* extracts were added to 30 µl of water, 25 µl total soluble plant proteins, or 20 µl total soluble protoplast proteins (kindly donated by Yaopan Mao, for extraction buffer see 3.2.7.2) (see Figure 3.9). Recombinant thrombin cleaved CBF1 protein is degraded in the presence of total soluble plant extracts and protoplast extracts (Figure 3.8, see lane 2 and 3 compared to lane 1). There appeared to be slightly more CBF1 protein present in the presence of protoplast extracts as compared to the plant extracts (lane 3) indicating that perhaps the buffer used to extract protoplasts resulted in less degradation than the other immunoprecipitation buffers used. When a second set of incubation experiments were conducted with recombinant CBF3, the protein was not degraded in the presence of protoplast extracts (data not shown). However, when immunoprecipitations were conducted with 20 µl ^{35}S –labelled, CBF1-overexpressing *E. coli* lysates spiked with 75 µl total soluble plant extracts, protoplast extracts or extraction buffer alone little or no CBF protein was detected (data not shown).

3.3.13 ^{35}S-METHIONINE LABELLED CBF1 IS NOT DETECTED BY IMMUNOPRECIPITATION OF PLANT EXTRACTS

Once the most useful extraction buffer was determined, an attempt was made to immunoprecipitate CBF1 from total soluble plant extracts. Plants were grown (see 3.2.2.2), labelled, and extracted as described (see 3.2.7.2). As seen in Figure 3.10, very faint but complex banding patterns were present in all lanes. No differences were detected between banding patterns generated by using pre-immune or immune sera (for

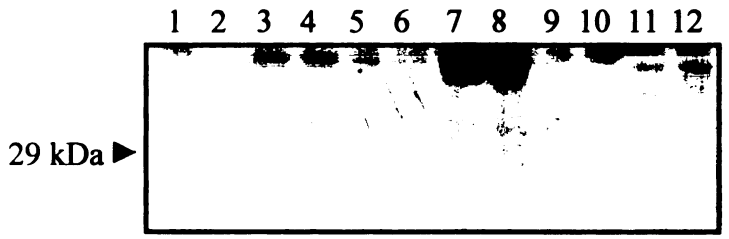

Figure 3.10. Immunoprecipitation of ^{35}S-methionine labelled CBF proteins from plant extracts

Immunoprecipitations were conducted using ^{35}S-methionine labelled plant tissue as described (see 3.2.11 #1 and #2). The plants types were nonacclimated (NA) and 24-h cold-acclimated (CA) A30a-1, A38b-7 (CBF3-overexpressing), and B16-1 (vector) (see 3.2.1). Immunoprecipitation of CBF1 was conducted with 20 µl of pre (pre)- or immune sera from N1 (N1) (see 3.2.5) and 45 µl of protein-A beads. The 29 kDa size marker, the predicted size of CBF1, is indicated with an arrow. The image shown is from a 4 hour exposure using an intensifying screen. The lanes are as follows:

1: N1, CA B16-1;
2: pre, CA B16-1;
3: N1, NA B16-1;
4: pre, NA B16-1;
5: N1, CA A38b-7;
6: pre, CA A38b-7;
7: N1, NA A38b-7;
8: pre, NA A38b-7;
9: N1, CA A30a-1;
10: pre, CA A30a-1;
11: N1, NA A30a-1;
12: pre, NA A30a-1.

example, compare lane 2 to lane 1, or lane 4 to lane 3). Additionally, the type of tissue used, (compare lanes 3, 7 and 11) or the state of acclimation (for example, compare lanes 1 and 3) did not seem to have an effect on the banding patterns. This experiment was repeated numerous times with similar results (data not shown). Overall, CBF proteins could not be clearly detected. The failure to detect the proteins was not due to the extraction buffer used as recombinant CBF1 protein was successfully immunoprecipitated in the protoplast buffer (data not shown). Immunoprecipitation experiments were also conducted using ^{35}S-methionine labelled Arabidopsis protoplasts (kindly donated by A. Sanderfoot). However, despite repeated attempts, incorporation of ^{35}S-methionine into total plant proteins remained low, possibly due to bacterial infection of the plants in tissue culture. In both cases, immunoprecipitations resulted in no visible banding patterns (data not shown).

3.3.14 CBF1 IS NOT DETECTED BY IMMUNOPRECIPITATION OF ^{32}P-ORTHOPHOSPHATE LABELLED PLANT EXTRACTS

Immunoprecipitations using ^{35}S-methionine labelled plants did not result in the detection of CBF1 proteins (see Figure 3.10A), possibly due to the low intensity of the ^{35}S signal. ^{32}P-orthophosphate produces a signal of higher intensity on x-ray film than ^{35}S-methionine, making it more desirable as a label (Harlow and Lane, 1988). Analysis of the CBF protein sequences indicated that they contain a total of 17 potential phosphorylation sites, including one putative MAP kinase site (Blom et al, 1999; E. Stockinger, M. Thomashow, unpublished). Therefore, plants were labelled with ^{32}P-

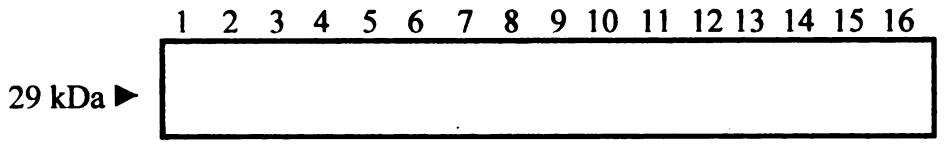

Figure 3.11. Immunoprecipitation of ^{32}P-orthophosphate labelled CBF proteins from plant extracts

Immunoprecipitation of CBF proteins from ^{32}P-orthophosphate labelled total soluble plant extracts. Immunoprecipitations were conducted as described (see 3.2.12) using 20 µl of pre (pre)- or immune sera from N2 (N2) (see 3.2.5) and 30 µl of protein-A beads. The plants types were nonacclimated (NA) and 6-h cold-acclimated (CA) WS, A30a-1 (CBF3-overexpressing), E71-1 (CBF2-overexpressing), and G7a-1 (CBF1-overexpressing) (see 3.2.1). The 29 kDa size marker, the predicted size of CBF1, is indicated with an arrow. The image shown is from a 21 day exposure using 2 intensifying screens. The lanes are as follows:

1: pre, NA WS;
2: N2, NA WS;
3: pre, CA WS;
4: N2, CA WS;
5: pre, NA A30a-1;
6: N2, NA A30a-1;
7: pre, CA A30a-1;
8: N2, CA A30a-1;
9: pre, NA E71-1;
10: N2, NA E71-1;
11: pre, CA E71-1;
12: N2, CA E71-1;
13: pre, NA G7a-1;
14: N2, NA G7a-1;
15: pre, CA G7a-1;
16: N2, CA G7a-1.

orthophosphate (see 3.2.12). No distinct bands were seen at ~29 kDa, the predicted molecular weight of the CBF proteins even after exposure to x-ray film with two intensifying screens for 21 days (see Figure 3.11). However, very faint bands are present in the lanes containing immunoprecipitations from nonacclimated WS (lane 2), A30a-1 (lane 6) and G7a-1 (lane 14) (see 3.2.1 for plant types). To confirm that the putative bands were indicative of real phosphorylation events, the experiment was repeated using the same types of tissue and the same protocols (data not shown). However, no distinct bands were seen and the CBF proteins could not be clearly visualized under any conditions. The failure to visualize the CBF proteins was not due to poor incorporation of ^{32}P-orthophosphate into total soluble proteins as visualization of 4 µl of radiolabelled protein showed distinct banding patterns indicating that the ^{32}P-orthophosphate had been incorporated into plant proteins (data not shown).

3.4 DISCUSSION

The regulation of a transcription factor can give many clues as to the regulation of the entire signal-transduction pathway of which it is a part. In the cold-acclimation signal transduction pathway, it had previously been determined that the CBF proteins are likely activators of the COR genes (Jaglo-Ottosen et al, 1998; Gilmour et al, 1998). Therefore, the CBF proteins appear to play key roles in initiating the changes in gene expression, such as activating the *COR* genes. Increasing our knowledge as to the level of CBF protein accumulation under nonacclimating and acclimating conditions, and whether the proteins are regulated by modification would increase understanding of the regulation of

the entire cold-induced signal transduction pathway.

Transcription factors, such as the *CBF* genes, can be regulated at many steps. They can be transcriptionally regulated or post-transcriptionally regulated, the polypeptide can be translationally regulated and post-translationally regulated through modification (Gallie, 1993; Schwechheimer et al, 1998). Transcription factors can also be regulated by their physical location in the cell (Gallie, 1993; Schwechheimer et al, 1998) or regulation can occur through a combination of some or all of the above. RNA analysis has indicated that *CBF* RNA accumulation increases within 15 min of a low temperature stimulus, peaks at 2-4 h of acclimation, and remains at a higher steady state level for as long as plants are under acclimating conditions (Gilmour et al, 1998). These data would indicate that the *CBF* genes are regulated in part, either transcriptionally, or post-transcriptionally by RNA stability. However, regulation through increased RNA accumulation under acclimating conditions does not rule out the possibility that the production and/or activity of the proteins are also translationally or post-translationally regulated under acclimating conditions as well.

One way to determine if the CBF proteins are translationally or post-translationally regulated is to examine the amount of protein and the location of the protein under both nonacclimating and acclimating conditions. Any changes in amounts or location of the CBF proteins would indicate if and how the proteins are regulated. Additionally, it would be useful to determine whether overexpression of *CBF1*, *CBF2* or *CBF3* resulted in changes in the amount of protein accumulation and/or the location of the protein under either condition. To answer these questions, the ability to recognize and detect the three CBF proteins was required. To this end, three types of anti-CBF1

antibodies were generated (see 3.2.5): those against full length recombinant CBF1 (F1 and F2, E. Stockinger, M. Thomashow, unpublished) those against an N-terminal peptide of CBF1 (N1 and N2) and those to a C-terminal peptide of CBF1 (C1 and C2). Peptides were selected as antigens to generate antibodies which should either detect CBF1 alone (N-terminal peptide) or all three CBF proteins (C-terminal peptide). The ability to distinguish between the CBF1 protein and all three CBF proteins combined would allow for detection of any differences in location or protein accumulation between CBF1 and the other CBF proteins.

Sequence analysis of the CBF2 protein indicates the presence of 17 potential phosphorylation sites; 9 on serine residues, 6 on threonine residues and 2 on tyrosine residues (http: //www.cbs.dtu.dk/services/NetPhos/-; Blom et al, 1999). Additionally, one of the sites, which is conserved with all three CBF proteins is a potential map kinase phosphorylation site (E. Stockinger, M. Thomashow, unpublished). This raised the possibility that the CBF proteins were post-translationally modified. Therefore, I wanted to determine if the CBF proteins are modified by a phosphorylation event under nonacclimating or acclimating conditions. Additionally, by investigating the phosphorylation state of CBF proteins under both conditions in transgenic plants, the mechanism by which overexpression of the CBF proteins activates the *COR* genes without a low temperature stimulus could be better understood.

Despite numerous and repeated attempts to detect the native CBF proteins in plants, the proteins were never definitively detected. There are many possible reasons as to the cause of the difficulties. The first reason could be that the anti-CBF1 antibodies were not effective at detecting the CBF proteins. This does not seem likely as all three

types of antisera were able to detect the recombinant CBF1 and CBF3 proteins in immunoblot analyses (see Figure 3.1, Figure 3.4).

One important and confounding factor associated with the creation of anti-CBF antibodies was with the pre-immune sera from all rabbits used. The pre-immune sera contained antibodies to many proteins in total soluble plant extracts (see Figure 3.2B) and also appeared to specifically recognize the CBF1 protein (see Figure 3.3 and Figure 3.4B). The recognition of CBF1 could very well be due to the AP2 DNA binding domain portion of the protein given that there are predicted to be ~90 AP2 domain containing proteins in the Arabidopsis genome alone (Riechmann and Meyerowitz, 1998). If other plant species, particularly those used to produce rabbit feed, also contain numerous AP2 DNA binding domain proteins, then it is very likely that rabbits are exposed to, and produce antibodies to, AP2 DNA binding domain proteins. Given that pre-immune serum is used as a negative control for immune serum, the presence of antibodies in the pre-immune serum that recognize CBF proteins could confound the interpretation of data. Additionally, in immunoprecipitation experiments, total soluble plant extracts are pre-cleared by incubating them with rabbit-serum bound to agarose beads (Sigma). If the rabbit serum contained antibodies that recognized the AP2 domain portion of the CBF proteins, it is possible that some or all of the native CBF proteins may have bound to these beads reducing the possibility of detecting the CBF proteins after immunoprecipitation.

One way to avoid the problem of anti-CBF or anti-AP2 domain antibodies in pre-immune serum would be to produce different types of antibodies. Monoclonal antibodies to the CBF proteins or CBF-specific peptides would be one way to eliminate antibodies

to other plant proteins. It is due to the high cost and technical difficulty involved with their production (Harlow and Lane, 1988) that monoclonal antibodies were not initially created. Another way to minimize the amount of non-specific plant protein antibodies in pre-immune serum would be to generate antibodies in another species, such as chickens. As chickens consume a different type of feed and have beaks instead of fleshy mouths like rabbits, they would presumably be exposed to far fewer plant proteins, and therefore make far fewer antibodies to plant proteins than rabbits.

Another possible difficulty in detecting the protein could be due to the fact that the protein is rapidly degraded. Regulation of transcription factors through conditional or constitutive degradation has been observed previously (Varshavsky, 1997). There is also evidence to support the idea that the CBF proteins are rapidly degraded. In all experiments where recombinant CBF1 was exposed to total soluble plant extracts, the protein was degraded, either partially or totally (see Figure 3.6B and Figure 3.9). This degradation occurred regardless of which extraction buffer was used, or if a complete mixture of protease inhibitors was added. Finally, the tight regulation of the *COR* genes could be an indication that the CBF proteins are rapidly degraded. If plants are removed from acclimating conditions and returned to warm temperatures, *COR* RNA accumulation decreases rapidly, and is virtually undetectable within eight h after a temperature shift (Hajela et al, 1990). This rapid decrease in RNA accumulation could be the result of the rapid degradation of the CBF proteins under nonacclimating conditions. However, until the protein is detected, whether or not it is rapidly degraded will remain unknown.

One interesting outcome of the ^{32}P-orthophosphate labelling experiments is the

possible indication that the CBF proteins may be phosphorylated under nonacclimating conditions (see Figure 3.11 lanes 2, 6 and 14). While the result was not repeatable, a faint signal was seen under nonacclimating conditions. If so, this would suggest that the CBF proteins are inactivated under nonacclimating conditions by a phosphorylation event. Inactivation of a transcription factor by phosphorylation is not uncommon. The phosphorylation event can prevent the import of the factor to the nucleus, as is seen with SW15, the binding of the factor to DNA, as is seen with Oct1 and Myogenin, or may prevent transactivation as is seen with ADR1 (Hunter and Karin, 1992). These data seem contrary to the evidence that protein kinases are activated under acclimating conditions in alfalfa (Jonak, 1996), if the data can be taken as an indication that homologous kinases are activated in Arabidopsis under acclimating conditions. However, it is possible that these kinases function to activate some other protein upstream of the CBF proteins, or proteins in another cold-induced pathway. One possibility is that under acclimating conditions, a cold-induced kinase functions to activate a phosphatase which then activates the CBF proteins by de-phosphorylation. However, until the experiment can be repeated, it will remain unknown as to whether or not the CBF proteins are actually phosphorylated under nonacclimating conditions.

While I was never able to detect the CBF proteins in plant extracts, I believe the effort to do so was worthwhile. In order to understand fully how the *CBF* genes function in the cold acclimation signal transduction pathway, the quantity and location of the proteins must be determined. Additionally, it would be of great importance and interest to determine if the proteins are regulated by modification under either nonacclimating or acclimating conditions. Without this information, complete understanding of the cold

acclimation pathway will not be possible. Despite the fact that these protein-related questions could not be definitively answered, they remain interesting questions and hopefully through some future effort, the answers will be resolved.

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4. CHAPTER 4: Overexpression of Arabidopsis *CBF1*, *CBF2* or *CBF3* in *Brassica napus* var. Westar results in increased BN gene expression and freezing tolerance

4.1 INTRODUCTION

Freezing temperatures, drought and other environmental stresses limit the geographical areas of crop production and are estimated to cause up to a 60% reduction in maximum crop yield annually (Levitt, 1980). Some plants withstand freezing temperatures by cold acclimating, the process by which plants increase in freezing tolerance after exposure to low, non-freezing temperatures. In 1970, Weiser proposed that, in addition to being associated with numerous physiological changes, such as increases in proline levels and total soluble sugars, cold acclimation is associated with changes in gene expression (Weiser, 1970). The identification of the *COR* (cold-regulated) genes in Arabidopsis -also called *LTI* (low temperature-induced), *KIN* (cold-inducible), *RD* (responsive to desiccation) and *ERD* (early dehydration-inducible)-, a small family of novel, highly hydrophilic polypeptides that are induced during both cold acclimation and drought stress, was direct evidence of such changes (Nordin et al, 1991; Wang et al, 1994; Welin et al, 1994), and indicated the presence of a cold and drought induced signal transduction pathway. Further research has shown that *COR* gene homologues are present in diverse freezing tolerant plant species, such as the *BN28* (Orr

et al, 1992) and *BN115* (Weretilnyk et al, 1993) genes in canola, the *wcs120* gene in wheat (Houde et al, 1992), the *HVA1* gene in barley (Heino et al, 1990, Hong et al, 1992) and the *cas15* gene in alfalfa (Monroy et al, 1993).

In Arabidopsis, the *COR* genes are transcriptionally regulated through a cold- and drought inducible promoter element, called the CRT/DRE (C-Repeat/Dehydration Responsive Element). The DRE element, TACCGACAT, was initially identified by Yamaguchi-Shinozaki and Shinozaki (1994) as being responsive to low temperature and dehydration. Later work showed that the core of the DRE, the CRT, CCGAC, was present in multiple copies in the promoters of *COR6.6* (Wang et al, 1995), *COR15* (Baker et al, 1994) and *COR78* (Horvath et al, 1993, Yamaguchi-Shinozaki and Shinozaki, 1993). Deletion analysis with the *COR15a* promoter showed that cold-induced activation of a reporter gene occurred when the CRT sequence was present in the promoter (Baker et al, 1994). Interestingly, this sequence is not only found in multiple copies in *COR* gene promoters in Arabidopsis, but also in the promoters of some of the cold induced genes in other species, such as the *BN115* gene in canola (Jiang et al, 1996), and the *wcs120* gene in wheat (Ouellet et al, 1998), giving rise to the hypothesis that cold and drought inducible gene expression may be highly conserved among different plant species.

A major breakthrough in understanding how plants sense and respond to low temperatures was the isolation of the *CBF/DREB* (CRT/DRE Binding Factors/Drought Response Element Binding factors) family of transcription factors, which bind to the CRT/DRE element and activate transcription under cold acclimating conditions (Stockinger et al, 1997; Gilmour et al, 1998; Liu et al, 1998; Shinwari et al, 1998). The three genes, *CBF1*, *CBF2* and *CBF3* have highly similar amino acid sequences (~85%)

contain AP2-like DNA binding domains, acidic activation domains, and have been shown to activate transcription in yeast (Stockinger et al, 1997; Gilmour et al, 1998). Constitutive overexpression of *CBF1*, *CBF2*, or *CBF3* in Arabidopsis results in constitutive *COR* gene expression and increased freezing tolerance under both acclimating and non-acclimating conditions (Jaglo-Ottosen et al, 1998; Liu et al, 1998; Gilmour et al, 2000; S. Gilmour and M. Thomashow, unpublished). The GenBank database contains several expressed sequence tags (ESTs) of sequences with a high level of similarity to the *CBF/DREB* family from diverse plant species such as canola, rice and tomato. This presents the possibility that homologous cold acclimation pathways exist in diverse plant species and creates the possibility of increasing freezing tolerance through genetic engineering.

Here, results are presented indicating that the *CBF*-activated signal transduction pathway is conserved in canola. An Expressed Sequence Tag (EST) with high similarity to the *CBF* genes in *B. napus* is present in GenBank. My results indicate that induction pattern for this putative *CBF* homologue from *B. napus* var. Westar, (*BnCBF*), is highly similar to that of the Arabidopsis *CBF* genes; transcript accumulation occurs within 15 min of a low-temperature stimulus and remains at a higher steady state level for at least 24 h of cold treatment. The increased *BnCBF* RNA levels are followed by increased expression of *COR* gene homologues (called *BN* genes) indicating the possibility of a *CBF*-induced signaling cascade similar to that seen in Arabidopsis (Thomashow, 1999). Additionally, constitutive overexpression of Arabidopsis *CBF1*, *CBF2* or *CBF3* in canola results in increased *BN* gene expression and increased freezing tolerance as compared to control plants under both non-acclimating and acclimating conditions. This increase was

seen in both cuttings and seedlings of transgenic canola plants. Additionally, as compared to control plants, *CBF*-overexpressing canola plants show increases in total soluble sugars under both nonacclimating and acclimating conditions, but significant increases in proline levels only under acclimating conditions. These data indicate that overexpression of the Arabidopsis *CBF* genes not only activates the *BN* genes, but also activates other known cold-acclimation inducible pathways such as those involved in increasing proline and total soluble sugars (see 1.4.5). These increases in proline and soluble sugars have also been seen in *CBF3*-overexpressing Arabidopsis plants and are associated with increases in freezing tolerance (Gilmour et al, 2000). I also investigated whether constitutive *CBF*-overexpression resulted in increased salt tolerance, as salt, drought and freezing stress all result in similar damage to plants (Thomashow, 1999; see 1.3). Additionally, overexpression of *CBF3* in Arabidopsis was found to result in increased freezing tolerance as well as increased tolerance to salt (Liu et al, 1998). However, I was not able to conclusively determine whether overexpression of the *CBF* family of transcription factor resulted in an increase in salt tolerance. Here I propose that the *CBF*-signal transduction pathway is conserved from Arabidopsis to its close relative, canola, and that overexpression of Arabidopsis *CBF* genes can increase freezing tolerance in the agronomically important crop, canola.

4.2 MATERIALS AND METHODS

4.2.1 PLANT GROWTH:

Brassica napus var. Westar seeds, a spring variety of canola, were planted in 10.5 x 10.5 or 12.5 x 12.5 cm pots containing Baccto Planting Mix (Michigan Peat, Houston, TX). One to five plants per pot were germinated in controlled environment chambers at 20-22° C under continuous cool white fluorescent illumination of 100-150 $\mu molm^{-2}s^{-1}$ light intensity as described (Gilmour et al, 1988). After ~2 weeks, plants were screened for the presence of the transgene (see 4.2.3 for details) and thinned to one plant per pot. Plants or cuttings were fertilized every 1-2 weeks with Peters 20-20-20, diluted as indicated by the manufacturer (Scotts, Chicago, IL). To induce cold acclimation, 4-6 week old plants were placed at 4° C under continuous fluorescent illumination of ~50 $\mu molm^{-2}s^{-1}$ for three weeks, or 4-6 week old cuttings were placed under the same conditions for two weeks. For the time course experiments, a total of 18 individual 4-6 week old wild-type plants were used. Tissue was collected from two individual plants at room temperature after which the remaining 16 individual plants were placed at 4° C under continuous fluorescent illumination of ~50 $\mu molm^{-2}s^{-1}$, and tissue was collected from two plants at each of the time points indicated. To analyze proline and sugar levels, plants were grown as above, with the exception that up to three plants were grown per pot.

4.2.2 TRANSFORMATION:

Canola plants that constitutively overexpress *CBF1*, *CBF2*, or *CBF3* were created by Susanne Kleff. Briefly, the cDNA sequence from each of the *CBF* genes were cloned into the pGA643 expression vector, which contains the NPTII reporter gene, under the control of the constitutive CaMV 35S promoter (An, 1987). *Agrobacterium tumefaciens* strain GV3101 was transformed with these plasmids by electroporation for use in cotyledonary petiole transformation (Maloney et al, 1989) into canola plants (kindly donated by W. Keller, Natl. Research Council, Canada and M. Moloney, University of Calgary, Canada).

Regenerated plants were analyzed for the presence of the T-DNA by detecting expression of the NPTII gene (encoding kanamycin resistance) using the NPTII ELISA kit (5 Prime – 3 Prime, Inc. Boulder, CO). Regenerated shoots which tested positive for the presence of the T-DNA were further analyzed for the presence of the *CBF* transcript and the expression of the cold-regulated genes, *BN115* and *BN28*. These T_0 plants were self-pollinated, and the T_1 generation seeds were collected and used for further studies.

4.2.3 TRANSGENIC PLANT SELECTION:

As plants in the T_1 generation are not homozygous for the T-DNA, all plants were tested for either the expression of the NPTII gene using the NPTII ELISA kit (5 Prime – 3 Prime, Inc. Boulder, CO) or for the presence of the NPTII gene using Polymerase Chain Reaction (PCR) before use in experiments. Primers were designed using the NPTII

gene sequence found in GenBank and were synthesized at the Michigan State University Macromolecular and Structure Facility (5': TGGAGAGGCTATTCGGCTA, 3': CACCATGATATTCGGCAAG). PCR was carried out in 25 µl reactions containing ~40 pM of each primer, ~10 ng of genomic DNA, {isolated using the Wizard^{R} genomic DNA purification kit (Promega, Madison, WI)}, 50µM dNTPs and 5% DMSO using a RoboCycler Gradient 96 Temperature Cycler (Stratagene, La Jolla, CA). Conditions were: 5 min at 94°C, then 30 cycles of: 1 min at 94°C, 1 min at 62°C, and 2 min at 72°C, followed by an additional 5 min at 72°C. The entire reaction mixture was combined with DNA loading buffer (Sambrook et al, 1989) and 0.1% ethidium bromide, visualized on 1% TBE agarose gels and checked for the presence of a ~600 bp band diagnostic of the NPTII gene.

4.2.4 CUTTINGS

For the experiments with cuttings, multiple plants from independent *CBF*-overexpressing T1 lines were analyzed for high levels of BN28 protein expression. Selection resulted in eight *CBF1* lines (#9, 10, 11, 21, 26, 47, 55, 97), seven *CBF2* lines (#40, 45, 53, 54, 65, 101, 113) and seven *CBF3* lines (#25, 87, 108, 120, 129, 130, 145). Three independent lines for each of the *CBF1*, *CBF2*, and *CBF3* constructs were selected and used to make cuttings: #9, 10 and 26 for *CBF1*; #45, 53 and 65 for *CBF2*; and, #25, 87 and 145 for *CBF3*. Additionally, cuttings were made of two independent vector lines, #23 and 161, as controls. To make the cuttings, leaves with petioles and some meristem

tissue were excised from plants with a razor blade, dipped into Bontone rooting powder (Bonide Products, Inc, Yorkville, NY) and placed in 8.5 x 8.5 cm pots containing moist soil. The pots were covered with plastic wrap, and set in controlled growth chambers under the conditions described above (see 4.2.1). After ~4 days the plastic wrap was slit and after another two days, it was completely removed. Cuttings were allowed to grow for 4-6 weeks or until new leaf tissue formed before being used in experiments.

4.2.5 SEEDLINGS

Seedlings from the three independent T1 lines generated by transformation of the *CBF1*, *CBF2* or *CBF3* constructs described above (see 4.2.4) were analyzed for further study. Additionally, five independent vector lines (23, 74, 161, 163 and 165), one line that no longer contained a copy of the vector, (28) and non-transformed wild type plants were used as controls. As above, all transgenic *CBF*-overexpressing, or vector control lines were analyzed for the presence of the NPTII gene either by ELISA or PCR before use in experiments (see 4.2.3).

4.2.6 RNA HYBRIDIZATION:

4.2.6.1 RNA isolation, northern transfer and hybridization

RNA was isolated using TRIZOL reagent (GibcoBRL, Grand Island, NY) as recommend by the manufacturer, except that ~200 mg canola tissue was ground to

powder with liquid N_2 prior to the addition of TRIZOL reagent. Northern transfers were prepared and hybridized as described (Stockinger et al, 1997). A total of10 μg of total RNA was used for hybridization with the *CBF1* and *BnCBF* probes and 5 μg total RNA was used when *BN115* or *BN28* were used as hybridization probes.

4.2.6.2 Generation of DNA probes

The hybridization probe for detecting all three Arabidopsis *CBF* transcripts was on the XhoI/XbaI fragment from pSJG6 (S. Gilmour, M. Thomashow, unpublished) which contains the full-length Arabidopsis *CBF1* coding sequence. Due to the high similarity of the three *CBF* genes, the *CBF1* probe hybridizes to transcript from all three *CBF* genes and was used to detect all three *CBF* transcripts. Plasmids containing *BN28* (Orr et al, 1992) and *BN115* (Weretilnyk et al, 1993) were kindly donated by Jas Singh. For hybridization probes, pBN28 and pBN115 were individually digested with EcoRI and the resulting full-length sequences were band isolated as described (Sambrook et al, 1989). The probe for *BnCBF* (the *Brassica napus CBF* homologue) was made by PCR amplification of the sequence from genomic *B. napus* var. Westar genomic DNA isolated using the Wizard^R genomic DNA purification kit (Promega, Madison, WI). Primers for the *BnCBF* gene (GenBank accession #AF084185) were created using the Primer Select program in DNAStar (Madison, WI) and were synthesized at the Michigan State University Macromolecular and Structure Facility (5': GGTTACGTTAGGCGGAGAGT, 3': GGACGGCGGCGGCAAAAG). PCR was carried out in 25 μl reactions containing

~40 pM of each primer, ~10 ng of genomic DNA, 50μM dNTPs and 5% DMSO using a RoboCycler Gradient 96 Temperature Cycler (Stratagene, La Jolla, CA). Conditions were: 2 min at 94°C then 30 cycles of: 1 min at 94°C, 1 min at 53°C, 1 min at 72°C' followed by an additional 5 min at 72°C. Total reactions were mixed with DNA loading buffer (Sambrook et al, 1989) and 0.1% ethidium bromide, visualized on 1% agarose TBE gels after which the fragment of interest was band isolated and purified as described (Sambrook et al, 1989).

4.2.6.3 Membrane washing and stripping

Membranes were washed as described (Stockinger, 1997). When required, membranes were stripped by adding boiling SDS buffer (0.1x SSPE and 0.5% SDS) and shaking at 80°C until no further radioactivity was detected on the membrane.

4.2.7 IMMUNOBLOT ANALYSIS

Total protein was extracted by grinding frozen tissue (about 300 mg) in 300 μl extraction buffer containing 50 mM Tris-HCl (pH 8.0), 5% glycerol, 100 mM KCl, 1.5% (wt/vol) polyvinyl-pyrrolidone, after which insoluble material was removed by centrifugation at 13,000 x g for 20 min at 4° C. The protein concentration in the supernatant was determined using the Bradford dye-binding assay (Bio-Rad, Hercules,

CA) and100 μg of total soluble protein per sample was fractionated by 10% tricine SDS/PAGE (Schägger and von Jagow, 1987), and transferred to 0.1 μm nitrocellulose membranes by electroblotting (Towbin et al, 1979) as described (Artus et al, 1996). BN28 protein was detected using antiserum kindly given by A. Johnson-Flanagan (diluted 1: 5000) (Boothe et al, 1995) and visualized using the ECL system (Amersham Buckinghamshire, UK).

4.2.8 PROLINE ANALYSIS

Proline was isolated using standard protocols as modified by Gilmour (Gilmour et al, 2000). Briefly, 50 mg of freeze-dried tissue harvested from 3-16 plants of each line were suspended in 5 ml of water at 80°C for 15 min. Samples were shaken for 1 h at room temperature then allowed to stand over night at 4°C. Samples were filtered through glass wool, after which proline levels in three replicates of each sample were measured by an acid nyhydrin assay. An unbalanced analysis of variance (ANOVA) of the samples was done using SAS PROC GLM [SAS Institute, SAS/STAT User's Guide, Version 6 (SAS Institute, Cary, NC, 1989)].

4.2.9 TOTAL SOLUBLE SUGAR ANALYSIS

Total soluble sugars were isolated using standard protocols as modified by Gilmour (Gilmour et al, 2000). Briefly, 50 mg of freeze-dried tissue harvested from 3-16

plants of each line were suspended in 5 ml of 80% ethanol at 80°C for 15 min. Samples were shaken for 1 h at room temperature then allowed to stand over night at 4°C.Three replicates of each sample were measured by a phenol sulfuric acid assay. An unbalanced ANOVA of the samples was conducted as described above (see 4.2.8).

4.2.10 ELECTROLYTE LEAKAGE ASSAYS

Electrolyte leakage freeze tests were conducted as described (Gilmour et al, 2000; see 2.2.6) with the modification that one individual plant or cutting was selected as a representative of a given transgenic line. Tissue was removed from all healthy looking leaves of a cutting or the smallest two leaves of a seedling using a 6-mm paper punch and three to four punches were used in each of the three replicate samples for each temperature point. Data shown is either that of individual cuttings or plants or the combined data of individual cuttings or plants. For the nonacclimated and acclimated seedling data, an unbalanced ANOVA of the combined leakage values at each given temperature was performed using SAS PROC GLM [SAS Institute, SAS/STAT User's Guide, Version 6 (SAS Institute, Cary, NC, 1989)]. The temperature at which 50% of electrolytes were leaked (EL_{50}) were determined as described in 2.2.6.

4.2.11 SALT STRESS EXPERIMENTS

In the first experiment, water was withheld from cuttings for 1 day prior to the addition of NaCl. Cuttings were placed in 150 mM NaCl for 7 days, rinsed, returned to

water and allowed to recover for four weeks before photographs were taken (Experiment conducted by S. Kleff). Seedlings were salt stress using one of two similar protocols. In the first protocol, seedlings were flushed with 1 l of 150 mM NaCl, then placed in trays containing 150 mM NaCl for six days. They were then flushed with 1 l 200 mM NaCl and placed in trays containing 200 mM NaCl for another seven days. Plants were then flushed with 1 l deionized water, placed in water containing Peters 20-20-20 as recommended by the manufacturer (Scotts, Chicago, IL) and allowed to recover for four weeks before photographs were taken. In the second protocol, seedlings were flushed with 1 l 200 mM NaCl and placed in trays containing 200 mM NaCl for ten days. Plants were then flushed with 1 l deionized water, placed in water containing Peters 20-20-20 as recommended by the manufacturer (Scotts, Chicago, IL) and allowed to recover for four weeks before photographs were taken.

4.3 RESULTS

4.3.1 THE B. NAPUS CBF *GENE HAS A SIMILAR INDUCTION PATTERN TO THE ARABIDOPSIS* CBF *GENES*

Amino acid sequences from the *CBF* genes were used to search the GenBank database for other sequences with high levels (greater than 50%) of similarity, . The sequence for an EST from *B. napus* encoding for a protein with high similarity to the CBF proteins was found (see Figure 4.1). To determine if this putative *CBF* homologue

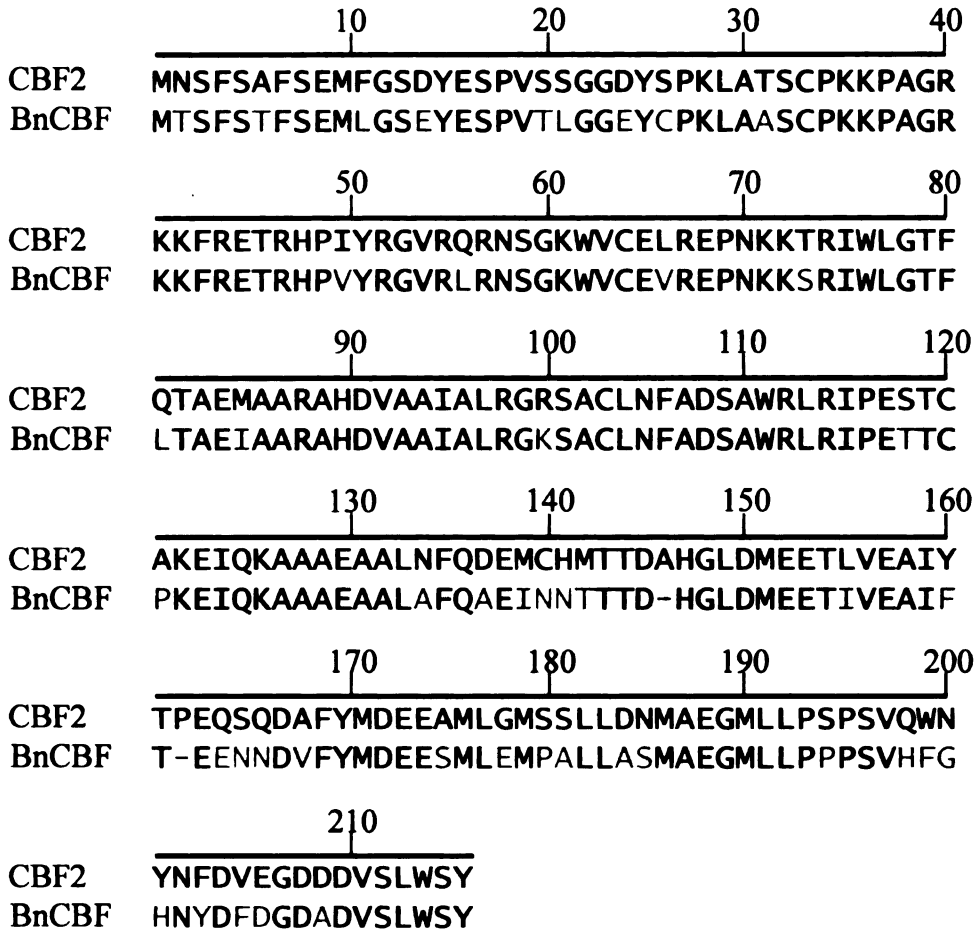


Figure 4.1. Amino acid sequence alignment of Arabidopsis CBF2 and BnCBF from *Brassica napus*

Alignment of the amino acid sequences from Arabidopsis CBF2 and BNCBF, the putative *CBF* homologue in canola. The amino acid sequences encoded for by CBF2 and BNCBF were aligned using Megalign (DNAstar Inc, Madison, WI). All divergent amino acids between the two sequences are indicated with orange. This image contains color.

from canola (*BnCBF*) was also cold induced, a time course of *BnCBF* RNA accumulation at 4°C was performed (Figure 4.2). Tissue was harvested from wild type canola plants under nonacclimating conditions and immediately frozen in liquid nitrogen after which the remaining plants were then put into acclimating conditions and tissue was harvested after 0.25, 0.5, 1, 2, 4, 12 and 24 h at 4°C (see 4.2.1). Total RNA was extracted and *BnCBF* RNA levels were analyzed by northern hybridization analysis (see 4.2.6). *BnCBF* RNA accumulation increased after 0.5 h at 4°C, reached a maximum level between two to four h at 4°C, and returned to a lower steady state level for up to 24 h at 4°C when the experiment was discontinued. To determine if the *COR* gene homologue, *BN115*, also had increased RNA accumulation under the same conditions, northern membranes were hybridized with a *BN115* probe (see 4.2.6). *BN115* RNA accumulation began to increase after ~4 h and continued to increase until the experiment was discontinued after 24 h. The *BN115* RNA increased ~ 3.5 h after the *BnCBF* RNA suggesting the presence of a *BnCBF*-induced signaling cascade in canola that is highly similar to that seen with the *CBF* and *COR* genes in Arabidopsis (Thomashow, 1999).

4.3.2 *GENERATION OF CANOLA PLANTS THAT CONSTIUTIVELY EXPRESS ARABIDOPSIS* CBF1, CBF2 *OR* CBF3

Overexpression of *CBF1*, *CBF2* or *CBF3* in Arabidopsis results in constitutive *COR* gene expression and increased freezing tolerance under both nonacclimating and acclimating conditions (Jaglo-Ottosen et al, 1998; Liu et al, 1998; Gilmour et al, 2000; S. Gilmour and M. Thomashow, unpublished). To determine if a similar increase in freezing

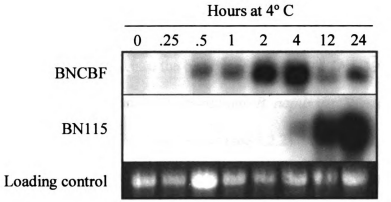


Figure 4.2. Time course of *BNCBF* and *BN115* RNA accumulation

BNCBF and *BN115* RNA accumulation. Total RNA was isolated from leaves of wild type canola plants acclimated at 4° C for the times indicated above the lanes (see 4.2.1 and 4.2.6.1). *BNCBF* and *BN115* transcripts were detected as described (see 4.2.6). The loading control panel is the ethidium bromide stained agarose gel containing RNA to which the *BNCBF* and *BN115* probes were hybridized in the panels above.

tolerance could be produced in an agronomically important species, canola, *B. napus* var. Westar plants that constitutively express the Arabidopsis *CBF1*, *CBF2* or *CBF3* genes were generated (see 4.2.2). Specifically, a cDNA copy of each individual Arabidopsis *CBF* was cloned into the binary vector pGA643 (An, 1987) under the control of the CaMV35S promoter and transformed into *B. napus* var. Westar plants using a cotyledonary petiole transformation method (see 4.2.2). In T_1 plants resulting from self-fertilized T_0 plants, immunoblot analysis indicated that nine lines, three lines for each *CBF*, had high levels of BN28 protein accumulation. These lines were selected for further analysis: #9, 10 and 26 for *CBF1*, #45, 53 and 65 for *CBF2* and #25, 87 and 145 for *CBF3* (data not shown). Additionally, five vector lines, which contained the pGA643 vector, #23, 74 and 161, 163 and 165, one transformed line that no longer contained a copy of the vector, #28, and non-transformed wild type plants were used for negative controls. As plants in the T_1 generation are not homozygous, all plants except for line #28 and wild type plants were tested for the presence of the T-DNA by assaying for the NPTII gene before use in experiments (see 4.2.3).

4.3.3 Overexpression of the Arabidopsis CBF *genes in canola resuts in increased* BN28 *and* BN115 *transcript accumulation*

The Arabidopsis *CBF* family of transcription factors can activate transcription of the *COR* genes in Arabidopsis (Jaglo-Ottosen et al, 1998; Gilmour et al, 1998; Liu et al, 1998; Gilmour et al, 2000). To determine if overexpression of the Arabidopsis *CBF* genes was able to initiate transcription of the *COR* gene homologue *BN28* in canola,

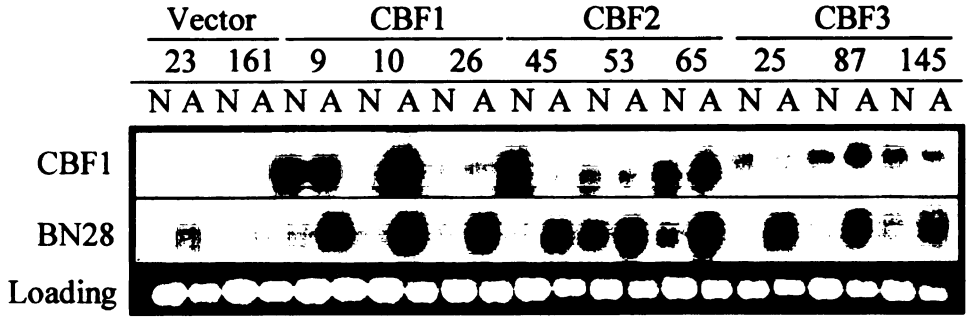


Figure 4.3. Accumulation of Arabidopsis *CBF* and *BN* RNA in individual canola cuttings

CBF and *BN28* RNA accumulation in individual nonacclimated (N) and two-week cold acclimated (A) cuttings. Total RNA was isolated from the transgenic cuttings indicated above the lanes as described (see 4.2.1 and 4.2.6.1). *CBF* and *BN28* transcripts were detected as described (see 4.2.6). The loading control panel is the ethidium bromide stained agarose gel containing RNA to which the *CBF* and *BN28* probes were hybridized in the panels above.

individual cuttings of the nine *CBF*-overexpressing lines were probed for accumulation of *CBF* and *BN28* transcripts under both acclimating and nonacclimating conditions (section 4.2.6, see Figure 4.3). The cDNA for *CBF1* was used as the hybridization probe to detect all three Arabidopsis *CBF* transcripts (see 4.2.6.2) but not the *BnCBF* transcript as the *CBF1* probe did not hybridize with *BnCBF* RNA to a significant extent under conditions used (S. Kleff, K. Jaglo and M. Thomashow, unpublished results). The levels of *CBF* RNA accumulation under nonacclimating and acclimating conditions did not seem to correlate well (i.e., in the *CBF1*-overexpressing transgenic line 10, RNA levels appear to increase under acclimating conditions; in the *CBF1*-overexpressing transgenic line 9, levels appear to be equal under both conditions; and in the *CBF2*-overexpressing transgenic line 45, RNA levels appear to decrease under acclimating conditions) indicating that individual cuttings may not have consistent *CBF*-transgene expression levels. To determine if the changes in *CBF* RNA levels isolated from the same individual plants were more consistent to levels seen in Arabidopsis, lower in the warm and higher in the cold, RNA was isolated from the same individual plants under both sets of conditions. Tissue from three to twelve individual plants from six transgenic and two control lines was isolated under nonacclimating and acclimating conditions and total RNA samples were probed for *CBF1*, *BN28* and *BN115* RNA accumulation (Figure 4.4). *CBF* transcript levels are increased in both nonacclimated and acclimated transgenic plants compared to control plants under identical conditions. *BN28* and *BN115* transcripts also accumulate to a greater extent in the *CBF*-overexpressing lines under nonacclimating conditions as compared to the control lines. However, in contrast to RNA accumulation under nonacclimating conditions, the amount of *BN115*

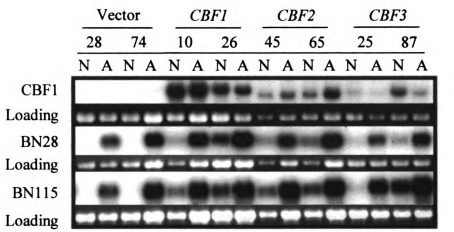


Figure 4.4. Accumulation of Arabidopsis *CBF* and *BN* RNA in pooled canola plants

CBF, *BN28* and *BN115* RNA accumulation in pooled nonacclimated (N) and three-week cold acclimated (A) plants. Total RNA was isolated from the pooled plants indicated above the lanes as described (see 4.2.1 and 4.2.6.1). The total number of pooled plants used to extract RNA was: 9 of #28, 7 of #74, 9 of #10, 9 of #26, 3 of #45, 7 of #65, 7 of #25 8 of #87. *BNCBF*, *BN28* and *BN115* transcripts were detected as described (see 4.2.6). The loading control panels are the ethidium bromide stained agarose gels containing RNA to which each probe was hybridized in the panel above.

and *BN28* transcript in acclimated transgenic plants does not appear to be greatly increased over that of acclimated control lines. This could indicate that maximum RNA accumulation is reached in the *CBF*-overexpressing lines under acclimating conditions.

4.3.4 Overexpression of the Arabidopsis CBF genes in canola resuts in increased BN28 protein accumulation

Overexpression of the Arabidopsis *CBF* genes results in increased *BN28* and *BN115* RNA accumulation (see Figure 4.3 and Figure 4.4). To determine if the levels of *BN28* transcript accumulation are representative of BN28 protein accumulation, protein extracts from individual nonacclimated and cold-acclimated *CBF*-overexpressing and control cuttings under were analyzed by immunoblot analysis (see 4.2.7) (Figure 4.5A). Under nonacclimating conditions, no protein accumulation is seen in extracts isolated from control cuttings. However, BN28 protein accumulation is observed in extracts from nonacclimated *CBF*-overexpressing lines, approximately equivalent to that of two-week cold-acclimated control cuttings, although there is variation between the lines. In extracts isolated from two-week cold-acclimated cuttings, there are increased levels of BN28 protein in transgenic cuttings as compared to the controls. A very similar pattern is seen when conducting the same experiments on nonacclimated and three-week cold-acclimated seed-grown plants (Figure 4.5B) indicating that overexpression of the Arabidopsis *CBF* genes results in increased BN28 protein accumulation under both nonacclimating and acclimating conditions, in both types of plant tissue.

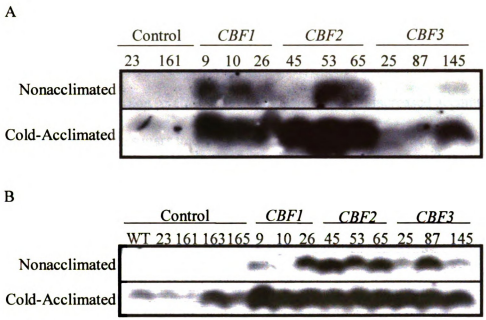


Figure 4.5. Accumulation of BN28 protein in individual *CBF*-overexpressing canola cuttings and plants

Total soluble protein was extracted from individual nonacclimated and cold-acclimated cuttings and seedlings grown as described (see 4.2.1 and 4.2.7). The amount of BN28 protein accumulation was determined by immunoblot analysis using 100 µg total soluble protein extracts and antiserum raised against the BN28 polypeptide (Boothe et al, 1995) as described (see 4.2.7).

A. BN28 protein accumulation in individual nonacclimated and two-week cold acclimated cuttings.

B. BN28 protein accumulation in individual nonacclimated and three-week cold acclimated seed grown plants.

4.3.5 Overexpression of the Arabidopsis CBF *genes in canola results in increased accumulation of proline and total soluble sugars*

Overexpression of *CBF3* in Arabidopsis results in increased accumulation of proline and total soluble sugars under both nonacclimating and acclimating conditions (Gilmour et al, 2000). To determine if there are similar increases in the accumulation of proline and soluble sugars in *CBF1- CBF2*-or *CBF3*-overexpressing canola plants, both types of small molecules were assayed. Figure 4.6 shows the graphical representation of the total amounts of free proline (upper graphs) and soluble sugars (lower graphs) under both nonacclimating (graphs on left) and acclimating (graphs on right) conditions in μg/mg dry tissue. None of the *CBF*-overexpressing lines have significant increases in proline as compared to control lines under nonacclimating conditions, while all three types of overexpressing lines have significant increases under acclimating conditions where $p<0.001$ (see 4.2.8). In regards to the amounts of total soluble sugars, the *CBF*-overexpressing lines all have different p values compared to the control lines under nonacclimating conditions. For *CBF1*, $p=0.056$, for *CBF2*, $p=0.018$ and for *CBF3*, $p=0.09$. Under acclimating conditions, all three *CBF*-overexpressing lines have significant increases in the amount of total soluble sugars as compared to control lines where $p<0.001$ (see 4.2.9). Table 4.1A shows proline accumulation in μg/mg of dry tissue from all *CBF*-overexpressing lines combined and all vector lines combined under both nonacclimating and acclimating conditions. Under nonacclimating conditions, the proline levels of transgenic plants are not significantly higher than those of control plants.

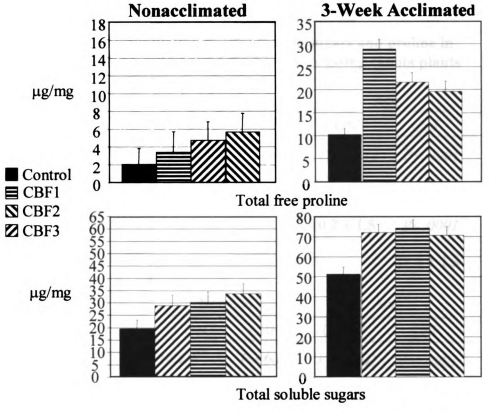


Figure 4.6. Accumulation of total soluble sugars and proline in control and *CBF1*, *CBF2* or *CBF3*-overexpressing canola plants

Pooled individuals of *CBF1*, *CBF2* and *CBF3*-overexpressing plants were used for both nonacclimated and 3-week cold-acclimated samples. Replicate samples from the same plant tissues were used for both the sugar (4.2.9) and proline (4.2.8) extractions. CBF1, CBF2 and CBF3 designate the combined results of *CBF1*- (9 of #10, 9 of #26); *CBF2*–(3 of #45, 7 of #65); and, *CBF3*- (7 of #25 8 of #87) overexpressing plants respectively. Control designates combined results from control plants (12 of WT, 9 of #28, 7 of #74). The amount of sugars or proline in µg/mg dry tissue are shown graphically. Error bars indicate standard error. The amount of free proline in *CBF*-overexpressing plants was not significantly different from control plants under nonacclimating conditions, whereas under acclimating conditions, all were significantly different at $p<.001$. The amount of total soluble sugars in CBF-overexpressing plants as compared to control plants had P values of .056 CBF1, .018 for CBF2 and .09 CBF3, whereas all the acclimated samples are significantly different at $p<.001$.

Table 4.1. Accumulation of total soluble sugars and proline in combined *CBF1*, *CBF2* and *CBF3*-overexpressing canola plants

A

		NonAcc		Acc	
		CONT	CBF	CONT	CBF
NonAcc	CONT	**2.1 ± 1.8**	*P=.1562*	*P =.0012*	*P<.0001*
	CBF		**5.5 ± 1.5**	*P=.0277*	*P<.0001*
Acc	CONT			**10.2 ± 1.5**	*P<.0001*
	CBF				**23.6 ± 1.3**

B

		NonAcc		Acc	
		CONT	CBF	CONT	CBF
NonAcc	CONT	**19.6 ± 3.3**	*P=.0089*	*P <.0001*	*P<.0001*
	CBF		**30.7 ± 2.4**	*P<.0001*	*P<.0001*
Acc	CONT			**51.3 ± 3.5**	*P<.0001*
	CBF				**72.4 ± 2.3**

Pooled individuals of *CBF1*, *CBF2* and *CBF3*-overexpressing plants were used for both nonacclimated and acclimated samples. Replicate samples from the same plant tissues were used for both the sugar and proline extractions. CBF designates combined results of all *CBF1*, *CBF2* and *CBF3* over expressing plants, (9 of #10, 9 of #26, 3 of #45, 7 of #65, 7 of #25 8 of #87). CONT designates combined results from all control plants (12 of WT, 9 of #28, 7 of #74). NonAcc designates nonacclimated and Acc designates three-week cold-acclimated. The amount of sugars or proline in μg /mg dry tissue ± standard error are represented on the diagonal in bold. P values for the comparisons of sugar or proline levels are indicated in the intersecting cells in italics.

A. Amount of proline in combined *CBF*-overexpressing and control plants.
B. Amount of total soluble sugars in combined *CBF*-overexpressing and control plants.

However, under acclimating conditions, there is a statistically significant increase in proline levels in transgenic plants as compared to control plants. Table 4.1B shows the total soluble sugar accumulation in μg/mg dry tissue from all *CBF*-overexpressing lines combined and all vector lines combined under both nonacclimating and acclimating conditions. The levels of total sugars are significantly higher in combined transgenic plants as compared to control plants under both temperature regimes.

4.3.6 Overexpression of Arabidopsis CBF *genes in canola results in increased freezing tolerance*

Overexpression of the three *CBF* genes in Arabidopsis results in increased freezing tolerance under both nonacclimating and acclimating conditions as determined by electrolyte leakage analysis (Jaglo-Ottosen et al, 1998; Liu et al, 1998; Gilmour et al, 2000; S. Gilmour and M. Thomashow, unpublished). To determine if overexpression of the Arabidopsis *CBF* genes in canola plants resulted in increased freezing tolerance, electrolyte leakage tests were conducted on both cuttings and seedlings, as seen in Figure 4.7A-E. Individual cuttings were used as representatives of transgenic lines and tissue was taken from all healthy looking leaves as described (see 4.2.10). Data shown are from one representative electrolyte leakage test using tissue from nonacclimated cuttings (Figure 4.7A) and two-week cold-acclimated cuttings (Figure 4.7B). Under both nonacclimating (Figure 4.7A) and two-week cold-acclimating conditions (Figure 4.7B), *CBF1*, *CBF2* and *CBF*3-overexpressing cuttings show a dramatic increase in freezing tolerance. For example, in Figure 4.7A, comparison of the amount of electrolytes leaked

Figure 4.7. Electrolyte leakage analysis of wild type and transgenic canola plants and cuttings

Leaves from nonacclimated and cold acclimated (see 4.2.1) cuttings (4.2.4) and seedlings (4.2.5), were frozen to the temperatures indicated and the extent of cellular damage was estimated by measuring electrolyte leakage as described (see 4.2.10). Error bars indicate the standard deviations of the three replicates of each data point unless otherwise indicated.

A. Electrolyte leakage analysis using individual nonacclimated cuttings of *CBF1* (#10), *CBF2* (#53) or *CBF3* (#145) overexpressing plants and two vector lines (#23 and #161). Color in this image.

B. Electrolyte leakage analysis using individual two-week cold-acclimated cuttings of *CBF1* (#10), *CBF2* (#53) or *CBF3* (#145) overexpressing plants and one vector line (#161). Color in this image.

C. Electrolyte leakage analysis using individual nonacclimated *CBF1* (#26), *CBF2* (#65) or *CBF3* (#87) overexpressing plants, two vector lines (#161 and #165) and WT plants. Color in this image.

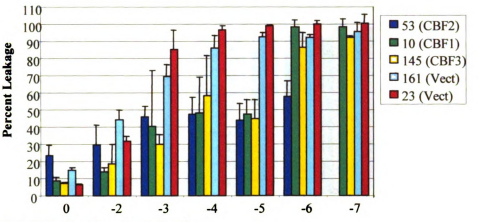
D. Electrolyte leakage analysis using individual three-week cold-acclimated *CBF1* (#10) or *CBF2* (#53) overexpressing plants, two vector lines (#23 and #161) and WT plants. Color in this image.

E. Electrolyte leakage analysis using combined data from all individual nonacclimated *CBF1*, *CBF2* or *CBF3* overexpressing plants (23 total) or control plants (10 total). Error bars indicate the standard deviation of all the replicates used at each data point. Except for 0^0 C, all temperatures are significantly different at $p<.01$ as determined by unbalanced one-way ANOVA (see 4.2.10).

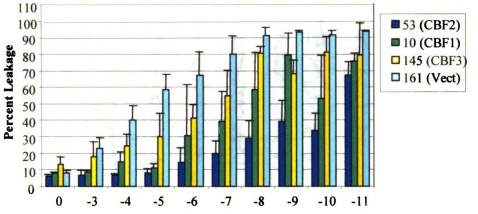
F. Electrolyte leakage analysis using combined data from all individual three-week cold-acclimated *CBF1*, *CBF2* or *CBF3* overexpressing plants (12 total) or control plants (8 total). Error bars indicate the standard deviation of all the replicates used at each data point. Except for 0^0 C, and -3^0 C, all temperatures are significantly different at $p<.01$ as determined by unbalanced one-way ANOVA (see 4.2.10).

Figure 4.7 cont

A. Individual nonacclimated cuttings



B. Individual cold-acclimated cuttings



C. Individual nonacclimated plants

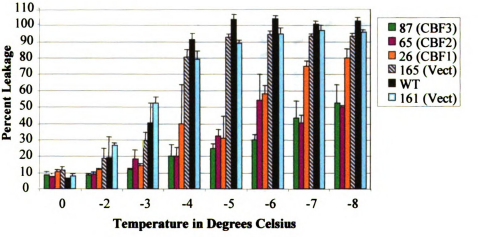
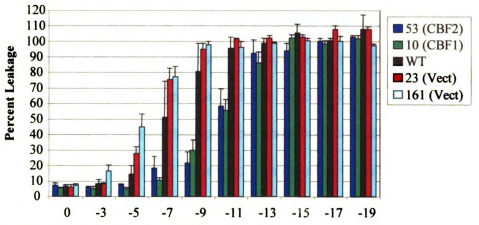
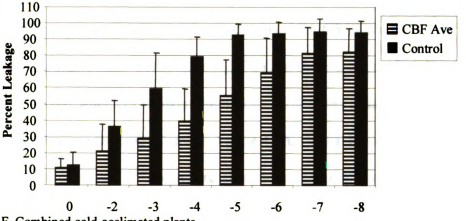


Figure 4.7 cont.

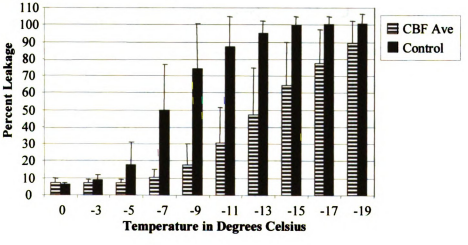
D. Individual cold-acclimated plants



E. Combined nonacclimated plants



F. Combined cold-acclimated plants



from the three *CBF*-overexpressing lines to that of the control lines at –5° C shows that the transgenic lines have ~42% leakage while the control lines have ~95% leakage. A similar comparison with two-week acclimated cuttings (7B) at–6° C shows ~20-40% leakage for transgenic plants and 65% leakage for the control line. These data indicate that significantly more membrane damage has occurred in the control lines than in the transgenic lines at the given temperatures under both nonacclimating and acclimating conditions. Figure 4.7 C shows one representative electrolyte leakage test using tissue from nonacclimated plants and Figure 4.7 D shows a representative electrolyte leakage test of three-week cold-acclimated plants. As seen with cuttings, there is a dramatic increase in freezing tolerance seen in *CBF1*, *CBF2* and *CBF*3-overexpressing plants under nonacclimating (7C) and three-week cold-acclimating (7D) conditions. To test whether overexpression of the *CBF* genes results in a statistically significant increase in freezing tolerance, Figure 4.7 E shows the combined data of all individual nonacclimated *CBF*-overexpressing plants (23 total) and all nonacclimated control plants (13). Figure 4.7 F shows the combined data for all *CBF*-overexpressing plants (12) and control lines (8) after three-weeks of cold-acclimation. Unbalanced one way analysis of variance (ANOVA) indicates that there is a statistically significant difference in electrolyte leakage over all temperatures tested except for at 0° C for nonacclimated plants, and a statistically significant difference in electrolyte leakage over all temperatures tested except at 0° C and –3° for three-week cold-acclimated plants where $t < 0.01$ (see 4.2.10). To quantitate the increase in freezing tolerance, EL_{50} values (the temperature at which 50% leakage is reached) were determined for *CBF*-overexpressing plants as described (see 4.2.10) and are shown in Table 4.2. When

Table 4.2. Comparison of EL_{50} values of combined *CBF1*, *CBF2* or *CBF3* overexpressing and control plants

		NonAcc		Acc	
		CONT	CBF	CONT	CBF
NonAcc	CONT	**-2.1 ± .34** (10)	*P<.0001*	*P <.0001*	*P<.0001*
	CBF		**-4.7 ± .40** (23)	*P<.0001*	*P<.0001*
Acc	CONT			**-8.1 ± .42** (8)	*P<.0001*
	CBF				**-12.7 ± .52** (12)

EL_{50} values were calculated using combined data from all individual nonacclimated and cold-acclimated *CBF1*, *CBF2* or *CBF3* overexpressing plants and compared ANOVA (see 4.2.10). EL_{50} ± standard error are represented on the diagonal in bold. P values are in italics and indicated in the intersecting cells, the number in the parenthesis indicates the number of individual plants combined. CONT designates combined control plants. CBF designates all combined *CBF1*, *CBF2* or *CBF3* overexpressing plants. NonAcc designates nonacclimated and Acc designates three-week cold-acclimated.

comparing EL_{50} values by ANOVA, all leakage amounts are significantly different from each other at $p < .0001$. Combined, these data clearly indicate that overexpression of Arabidopsis *CBF1*, *CBF2* or *CBF3* in canola results in a significant increase in freezing tolerance.

4.3.7 Osmotic stress tolerance of CBF1, CBF2 or CBF3 overexpressing canola lines

Freezing, drought, and osmotic stress all result in cellular dehydration and cause damage to membranes in plants (Thomashow, 1999; see 1.3). Additionally, Liu et al (1998) found that overexpression of *CBF3* in Arabidopsis resulted in increased freezing, drought and osmotic stress tolerance. As overexpression of the Arabidopsis *CBF* genes in canola results in increased freezing tolerance (see 4.3.6), it was of interest to determine if it also resulted in increased tolerance to salt. Plants and cuttings were exposed to 150 mM-200mM NaCl for 7 to 13 days, returned to deionized water and observed for recovery as described (see 4.2.11). While *CBF*-overexpressing plants and cuttings had increased survival after salt stress compared to control plants and cuttings in two experiments (Table 4.3) no differences in survival between transgenic and control plants were seen in the third experiment (Table 4.3). Further experiments need to be conducted to determine if overexpression of the Arabidopsis *CBF* genes in canola results in increased tolerance to salt.

Table 4.3. Survival of *CBF*-overexpressing and control cuttings and plants after salt stress.

	Experiment number		
	1	2	3
CBF1	2/4	3/7	1/5
CBF2	4/7	4/5	2/2
CBF3	5/6	4/6	4/6
Total CBF	11/17	11/18	7/13
control	0/3	0/7	8/14

Survival of CBF-overexpressing plants and cuttings after salt stress. Cuttings (experiment 1) and seedlings (experiments 2 and 3) were subjected to 150 mM to 200mM NaCl for 7-13 days as described (see 4.2.11). Recovery after salt stress was determined approximately 1 month after the removal of the salt stress. Cuttings and seedlings were considered "recovered" if tissue remained green and growth continued in the month following the removal of the osmotic stress. The numbers shown in the table are the number of individuals that recovered over the total number of individuals salt stressed.
CBF1, *CBF2* and *CBF3*-overexpressing plants and cuttings used are those described in 4.2.4 and 4.2.5. Control lines include all lines in 4.2.4 and 4.2.5 and wild type plants.

4.3.8 OVEREXPRESSION OF CBF1, CBF2 *OR* CBF3 *IN CANOLA DOES NOT CAUSE GROSS PHENOTYPIC CHANGES*

Overexpression of *CBF1*, *CBF2* or *CBF3* can cause a dwarf phenotype in *CBF*-overexpressing Arabidopsis plants with high transgene expression levels (Liu et al, 1998; Gilmour et al, 2000; A. Sebolt, S. Gilmour, M. Thomashow, unpublished). To determine if overexpression of the Arabidopsis *CBF* genes had an effect on the phenotype of canola plants, plants were grown to 27 days of age and photographs of representative plants were taken (Figure 4.8). There were no gross phenotypic differences observed between transgenic and control plants under the described growing conditions (see 4.2.1) with the exception that some transgenic plants appear to be a darker shade of bluish-green than control plants. The basis for this difference is not known. Additionally, when transgenic plants were grown from seeds to maturity, no dramatic changes in the overall life cycle of plants or time to flowering were observed. It should be noted that plants were grown in growth chambers under 24 h of artificial light (see 4.2.1). These conditions may not result in phenotypes representative of those seen by plants grown in the field.

4.4 DISCUSSION

The recent sequencing of the Arabidopsis genome suggests that there are many AP2 domain-containing proteins. Estimates indicate that there may be 90 AP2 domain-containing proteins in all (Riechmann and Meyerowitz, 1998). Sequencing efforts in other plant species have shown that AP2 domain containing proteins are conserved in

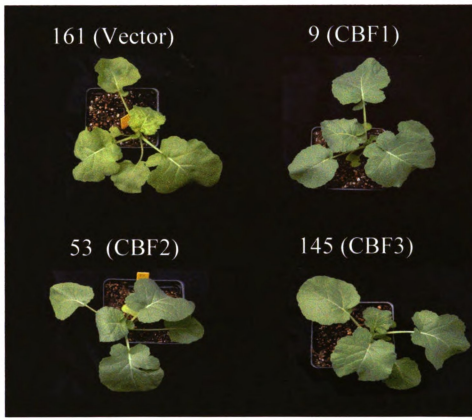


Figure 4.8. Photograph of transgenic *CBF*-overexpressing and control canola plants

One representative plant grown as described (see 4.2.1) of each *CBF*-overexpressing line is shown at 27 days of age: #9 for *CBF1*, #53 for *CBF2* and #145 for *CBF3*. As a comparison, the vector control line #161 is shown. No gross phenotypic differences were observed under the growing conditions used between *CBF*-overexpressing plants and the vector lines except that *CBF*-overexpressing plants sometimes appeared to be a slightly darker shade of bluish green than control plants. This image is in color.

angiosperms and have been identified in both monocots and dicots (Riechmann and Meyerowitz, 1998). In fact, when searching the entire database, sequences significantly similar to the Arabidopsis *CBF1* AP2 domain were found, including sequences in *Oryza sativa*, *Prunus armeniaca*, *Catharanthus roseus*, *Solanum tuberosum*, *Nicotiana sylvestris*, *Stylosanthes hamata* and others. Presumably more exist and will continue to be found as sequencing efforts advance in other plant species. AP2 domains are DNA-binding domains first identified in the APETALA2 protein that, to date, have only been found in plants (Riechmann and Meyerowitz, 1998). While not all of the large family of AP2 domain-containing proteins have been assigned functions, many, if not all, of the characterized proteins appear to play regulatory roles: APETALA2 in flower development (Jofuku et al, 1994); TINY in plant cell size (Wilson et al, 1996); atERFs in ethylene signaling in Arabidopsis (Hao et al, 1998; Fujimoto et al, 2000); EREBS in ethylene signaling in tobacco (Ohme-Tagaki and Shinshi, 1995); and the *CBF* genes in activating cold regulated gene induction (Stockinger et al, 1997; Liu et al, 1998; Jaglo-Ottosen et al, 1998; Gilmour et al, 1998; Medina et al, 1999; Gilmour et al, 2000) to name a few.

When generating a dendrogram of all AP2 domain-containing proteins in GenBank using Clustalx, a subset of proteins were found to cluster closely with the *CBF*-cold induced proteins in Arabidopsis indicating the possibility of functional similarity (T. Wagner, K. Amundsen, M. Thomashow, unpublished). Indeed, when the cold-acclimation induction pattern of the putative *CBF* homologues in canola, wheat and rye were investigated, a similar pattern to that seen in Arabidopsis was observed: little or no RNA is present under nonacclimating conditions, after ~30 min RNA accumulation

increases and remains at a higher steady state level for a least 24 h of acclimation (see 4.3.1; K. Amundsen, M. Thomashow, unpublished). If the *COR* gene homologues, *BN28* in canola and *COR39* in wheat and rye, are then used to probe the same RNA samples, a delayed increase in RNA accumulation is seen after ~ 4hrs, strongly indicating that the same cold-acclimation pathway present in Arabidopsis may be present in agronomically important crop species (K. Amundsen, M. Thomashow, unpublished). This potential conservation of induction pathways in diverse plant species is exciting for two reasons: 1) it suggests that the CBF pathway may have evolved before the crop species diverged, and; 2) it suggests the possibility that overexpression of the *CBF* genes and *CBF*-homologues in different crops species may be a viable way to increase freezing tolerance of agronomic crops.

To a limited extent, the *CBF* genes from closely related species appear to be interchangeable. Overexpression of the Arabidopsis *CBF* genes in canola resulted in increased expression of the *BN28* and *BN115* genes (Figure 4.3, Figure 4.4 and Figure 4.5), a statistically significant increase in the accumulation of soluble sugars under both nonacclimating and acclimating conditions (Figure 4.6 and Table 4.1A), and a statistically significant increase proline levels under cold acclimating conditions (Figure 4.6 and Table 4.1B). Additionally, when assayed for increased freezing tolerance by electrolyte leakage assays, a statistically significant increase in freezing tolerance was seen under both nonacclimating and acclimating conditions, as determined by comparing the temperature at which 50% leakage occurs, EL_{50} (Table 4.2). The increase in freezing tolerance was also seen when comparing leakage of all *CBF*-overexpressing plants to control plants over all temperatures tested except for at 0° C for nonacclimated plants,

and at 0° C and –3° C for acclimated plants (see Figure 4.7E and 4.7F).

Despite the fact that the *CBF* genes were under the control of the constitutive 35S promoter, there appears to be a "super induction" of the downstream genes in the *CBF* regulon under acclimation conditions. This induction was seen by the increase in BN28 protein accumulation in acclimated *CBF*-overexpressing plants as compared to control plants (Figure 4.5), and a statistically significant increase in proline accumulation seen only under acclimating conditions (Figure 4.6 and Table 4.1B). This superinduction could be due to the cumulative effect of the induction of the endogenous *BnCBF* genes in addition to the Arabidopsis *CBF* genes under the control of the CaMV 35S promoter, increased binding or activation of CBF proteins under acclimating conditions (Kanaya et al, 1999). Alternatively, it could be due to increased RNA stability of the *BN* genes and/or the genes involved in proline accumulation such as *P5CS* (Hare et al, 1999) under acclimating conditions. This increase in gene expression also appears to result in an additional increase in freezing tolerance as there is a ~2.5° C increase in the EL_{50} of nonacclimated transgenic plants compared to control plants whereas there is a ~4.5° C increase in the EL_{50} of acclimated transgenic plants compared to control plants. This could again be a cumulative effect of the transgene-induced *BN* gene expression in addition to the endogenous *BnCBF* induced *BN* gene expression. The statistically significant increase in proline levels under acclimating conditions could also be due to the "super induction" or, alternatively, there could be a significant increase in proline under nonacclimating conditions, but larger sample sizes and/or homozygous lines would need to be used to demonstrate the difference.

Interestingly, a high correlation has been seen between electrolyte leakage tests

and winter survival in different varieties of winter and spring canola under acclimating conditions (Teutonico et al, 1993; Song and Copeland, 1994). If this correlation is applicable to acclimated *CBF*-overexpressing transgenic plants, then I could anticipate that field trials will show an increase in freezing tolerance/winter survival of *CBF*-overexpressing plants as compared to control plants. However, this difference remains to be tested.

Despite the fact that freezing tolerance, drought tolerance and salt tolerance are interrelated (Thomashow, 1999), and *CBF3*-overexpression in Arabidopsis resulted in increased freezing, drought and osmotic stress tolerance (Liu et al, 1998) I could not detect a significant increase in salt tolerance between *CBF*-overexpressing and control plants (Table 4.3). While there was a significant increase in survival after salt stress observed in the first two experiments, the difference was not observable in the third experiment. Whether this was due to the differences in how the experiments were conducted, the differences in expression in the individual plants used in each experiment, or some other factor remains to be determined. By doing large scale experiments with larger sample sizes or perhaps even field trials with homozygous lines, it should be possible to determine definitively whether or not overexpression of the Arabidopsis *CBF* genes in canola results in increased salt tolerance under nonacclimating conditions.

Overexpression of Arabidopsis *CBF1, CBF2* or *CBF3* in canola plants did not result in gross phenotypic changes in the investigated lines (Figure 4.8). Overexpression of *CBF1* in Arabidopsis ecotype RLD also did not result in gross phenotypic changes in the lines investigated (Jaglo-Ottosen et al, 1998). However, overexpression of *CBF1, CBF2* or *CBF3* in the WS ecotype (Gilmour et al, 2000; A. Sebolt, S. Gilmour, M.

Thomashow, unpublished) and overexpression of *CBF3* in the Columbia ecotype (Liu et al, 1998) resulted in a dwarf phenotype and delayed growth in some homozygous lines. While it appears that there is a correlation between levels of transgene expression and the slow growth/dwarf phenotype (Liu et al, 1998; S. Gilmour, M. Thomashow, unpublished) the ultimate cause of these phenotypic changes remains unknown. If it is due to high levels of gene expression, then perhaps none of the recovered *CBF*-overexpressing canola lines have an expression level high enough to induce phenotypic changes. Alternatively, the phenotypic changes could be species and/or ecotype specific, or may only occur if the endogenous *CBF* genes are overexpressed, not if *CBF* genes from another species are overexpressed. Finally, the conditions in which the plants are grown could have an effect on whether phenotypic changes are seen. The exact reason(s) for the dramatic change in phenotype seen in the Arabidopsis plants, however, remain to be determined.

Given that *CBF*-like genes appear to be present in a variety of plant species, including chilling sensitive plants, such as tomato and rice (http: //www.ncbi.nim.nih.gov: 80/BLAST; Altschul et al, 1997) it will be interesting to determine if the *CBF* homologues in those species are also cold regulated. Data from Tanksley's lab indicate that when looking at complex quantitative traits, it is often the regulation of the critical transcription factor(s) that change, and not the presence or absence of the transcription factor itself that cause the differences in phenotype (Frary et al, 2000). If this is correct for the *CBF* genes, then chilling sensitive plants (plants that do not acclimate well) may still contain the *CBF* transcription factors, as is supported by sequence data, but the regulation of the gene(s) has been changed or lost. If chilling and

freezing sensitive plants do contain *CBF* and *COR* gene homologues, then genetic engineering by overexpressing the *CBF* genes has the potential of being highly successful in these species. By combining traditional plant breeding and genetic engineering, the long term goal of increasing freezing tolerance may finally be realized.

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5. CONCLUSIONS

This thesis reports on the roles of the Arabidopsis *CBF* family of transcription factors in cold acclimation. When *CBF1* was first identified by Stockinger et al (1997), it was unclear if the protein encoded by the gene played a role in the activation of *COR* genes in plants. Additionally, it was unknown if all the characterized *COR* genes would be activated, whether other uncharacterized *COR* genes would be activated, and if the activation of the battery of *COR* genes would increase freezing tolerance without a low temperature stimulus. The work done in Chapter 2 clearly shows that the CBF1 transcription factor can activate transcription of all of the known *COR* genes without a low temperature stimulus and that this activation results in an increase in freezing tolerance which was seen by electrolyte leakage analysis and whole plant freeze tests.

While the data presented in Chapter 2 show that overexpression of *CBF1* results in increased *CBF1* RNA accumulation, they did not address whether there was also increased accumulation of the CBF1 protein. Additionally, all of the regulation data on the *CBF* family of transcription factors to date is based on RNA accumulation of the genes under varying conditions. To truly understand how the gene is regulated, information as to the amount of CBF protein that is accumulated under nonacclimating and acclimating conditions is critical. While RNA accumulation data may give an indication as to the amount of protein that can be produced, it does not necessary reflect the actual amount of protein produced, or the activity of the protein. To answer these questions, the amount of protein that is accumulated, the location of the protein under

differing environmental conditions, and whether the protein is modified to regulate its activity must be determined. Despite repeated efforts to detect the CBF proteins in plant tissue, I was never successful in this endeavor.

Given that overexpression of each of the three *CBF* genes in Arabidopsis results in increased freezing tolerance, I wanted to address the question if overexpression of the three *CBF* genes in an agronomically important species also results in increased freezing tolerance. To this end, Susanne Kleff generated transgenic *Brassica napus* var. Westar plants, a close relative of Arabidopsis, that constitutively overexpress *CBF1*, *CBF2* or *CBF3*. Chapter 4 shows that the Arabidopsis CBF proteins can activate expression of the Brassica *COR* gene homologues, the *BN* genes, and also results in increased freezing tolerance. This increase in freezing tolerance was seen under both nonacclimating and acclimating conditions, with a more dramatic increase seen under acclimating conditions. A definitive increase in salt tolerance was not detected, although two out of three experiments showed that *CBF*-overexpressing canola plants had increased survival after salt stress as compared to control plants.

Overall, the data indicate that the *CBF* family of transcription factors play important roles in cold acclimation. Overexpression of *CBF1* can activate the *COR* genes and increase freezing tolerance without a low temperature stimulus in Arabidopsis, and overexpression of all three of the *CBF* genes in canola results in an increase in freezing tolerance under both nonacclimating and acclimating conditions. While these data are informative, there are still many interesting questions that remain to be asked.

One key question is: to what level do the CBF proteins accumulate under acclimating and nonacclimating conditions? Where are the CBF proteins located under

nonacclimating and acclimating conditions? Are the proteins sequestered from the nucleus under nonacclimating conditions, and if so, is a modification event involved? How does constitutive overexpression of the genes effect the accumulation of the protein in transgenic plants? While I was not able to successfully answer these questions, this does not mean that the questions cannot be answered, nor does it mean that future efforts should not be directed towards answering these questions. Due to the many problems associated with the detection of the protein using polyclonal antibodies, I believe that future efforts should involve the use of monoclonal antibodies. This change in antiserum should greatly reduce problems with cross hybridization to other plant proteins and should increase detection of the CBF proteins. Additionally, creating transgenic plants with tagged CBF proteins may aid in the detection of the protein. If the protein is rapidly degraded, and the data in Chapter 3 support this hypothesis, then adding a large tag to the protein may slow or stop the degradation process. While this would not aid in detecting native CBF proteins, it could elucidate the location of the chimeric protein and whether the protein is modified, both of which are important clues as to the regulation of the native proteins.

Another interesting question that remains to be answered is how many genes are activated by the CBF proteins? All of the characterized *COR* genes are activated by overexpression of the *CBF* genes, but how many more as of yet uncharacterized genes with CRT/DRE sequences in their promoters are also activated? There is now good evidence that overexpression of *CBF3* in Arabidopsis results in activation of *P5CS*, a gene involved in proline production, and the increase in total soluble sugars in *CBF*-overexpressing canola plants would indicate that changes in the metabolism of sugars are

also regulated by the CBF proteins. However, which of these changes in gene expression are direct effects of *CBF*-overexpression and which are indirect effects is not currently known.

One way to address these questions would be to analyze the total changes in RNA accumulation in *CBF*-overexpressing and control plants under various conditions. This could be done using microarray analysis. By looking at the profiles of the changes in gene expression in the *CBF*-overexpressing plants as compared to control plants, the number of genes activated and the level of activation induced by *CBF* expression could be determined. By comparing the genes activated in *CBF*-overexpressing plants to both nonacclimated and acclimated control plants, the number of genes directly and indirectly activated by CBF proteins, and the percentage of those genes to the total number of genes activated by cold acclimation could be determined. After establishing which changes in gene expression that occur in *CBF*-overexpressing plants reflect the changes that occur during cold acclimation, a time-course of the genes activated by cold acclimation in control plants could be conducted. The cascade of gene activation in control plants could then be compared to the genes activated in *CBF*-overexpressing plants. If the overexpressing plants have increased activation of both "early" induced and "late" induced genes, this could give an indication that activation of the "late" genes is an indirect effect of *CBF*-overexpression.

There are also more questions to be addressed in the *CBF*-overexpressing canola lines. While the data in Chapter 4 indicate that the transgenic canola plants have increased freezing tolerance under controlled lab conditions, they do not address how well the plants survive in the field, or if overexpression of the *CBF* genes results in

deleterious effects in terms of yield and oil quality. To fully characterize these lines, rigorous field testing needs to be conducted. The overall phenotype of the plants, the tolerance to osmotic and freezing stress as well as other types of stress, the oil quality and the yield of *CBF*-overexpressing lines all need to be determined before it can be established whether or not overexpression of the *CBF* genes is a viable solution to increasing the freezing tolerance of canola plants.

The last set of questions that are only briefly addressed in this thesis are what is the extent of conservation of the *CBF* signal transduction pathway? How may different types of plant species contain *CBF* homologues? If homologues are contained in chilling sensitive species, and preliminary data indicate that they do, then how are the *CBF* genes regulated in these species? Do *COR* gene homologues exist in chilling tolerant species, if so, do they contain CRT/DRE sequences in their promoters? By continuing to search for and characterize *CBF* homologues in diverse plant species, the answers to these questions should become apparent. If there is a high level of conservation in diverse plant species, then manipulation of the expression of the *CBF* family of transcription factors may be a viable means of increasing freezing tolerance in many important agronomic crops.