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By

Daniel D. Cook

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ABSTRACT

Molecular and Metabolic Responses to Dehydration and Cold Acclimation

By

Daniel D. Cook

Plants respond to the environment through changes in gene expression, proteins, and metabolites. In many species these changes allow a plant to cope with the environment. One such example is cold acclimation, a dynamic process by which plants exposed to low non-freezing temperatures increase their freezing tolerance. This dissertation focuses on two related projects; investigations of molecular responses to dehydration and metabolic and molecular responses to cold acclimation.

In response to low temperature and dehydration, the model plant Arabidopsis activates a set of genes whose promoters contain the C-repeat / dehydration responsive element. A family of transcription factors, CBF/DREB1, has been identified that are important regulators of these genes in response to low temperature. We identified a homolog, CBF4, of the CBF/DREB1 family of transcriptional activators. Unlike the CBF/DREB1 proteins, *CBF4* is responsive to drought and abscisic acid but not to low temperature. Furthermore, over-expression of CBF4 in transgenic Arabidopsis plants activates genes containing the C-repeat / dehydration responsive element. In addition, like transgenic plants over-expressing CBF/DREB1, plants over-expressing CBF4 were also found to be more freezing and drought tolerant. Thus CBF4 may be responsible for activating the CBF regulon in response to drought.

In addition to changes in gene expression, Arabidopsis also increases levels of soluble sugars and proline in response to cold acclimation. Over-expression of the CBF/DREB1 proteins results in accumulation of these metabolites. In order to investigate the global metabolic changes associated with cold acclimation, we profiled the metabolomes of non-acclimated, cold-acclimated, and transgenic plants over-expressing CBF3. In response to cold acclimation there is a significant increase in a large number of metabolites. The majority of the metabolites that increase due to cold acclimation are also mimicked by CBF3 over-expression. Significantly, natural variation in freezing tolerance between Arabidopsis ecotypes is reflected in the accumulation of many cold responsive transcripts and metabolites. A significant number of the cold responsive metabolites that differ in their accumulation between cold acclimated Arabidopsis ecotypes were also influenced by CBF over-expression. We propose that CBF is responsible for much of the natural variation in the level of metabolite accumulation in cold acclimated Arabidopsis ecotypes Ws and Cvi.

Furthermore we investigated whether circadian rhythms and the time plants are transferred to low temperature would influence *CBF* induction by low temperature. We found that the levels of *CBF* induction in response to low temperature were modulated by circadian rhythms. In addition we found that a transcription factor *RAV1* showed a similar induction response to low temperature as *CBF* due to circadian rhythms. We propose that this gating phenomena requires first, that the gene shows circadian regulated cycling and second, that the gene is immediately downstream of the low temperature stimulus. We propose that this gating phenomenon is modulated by calcium levels which also show a basal circadian cycling.

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Preface

In Chapter 2, the experiments were conducted by the author of this thesis and Volker Haake at Mendel Biotechnology. Volker Haake was responsible for making the transgenic plants and the evaluation of these plants in the assays for drought and freezing tolerance and the figures associated with this data. The results from this chapter were published in Plant Physiology.

In Chapter 3, the experiments were conducted by the author of this thesis at the Max Planck Institute of Molecular Plant Physiology in the laboratory of Dr. Oliver Fiehn and at Michigan State University in the laboratory of Dr. Mike Thomashow. I would specifically like to thank Anne Eckhardt and Vladimir Tolstikov at the Max Planck Institute of Molecular Plant Physiology for their operation of the GCMS (gas chromatograph mass spectrometer) and LCMS (liquid chromatograph mass spectrometer). Their work and time was essential for the completion of this work. Furthermore I would like to thank Oliver Fiehn for his help with the project as well as allowing me to perform these experiments in his laboratory.

In chapter 4, all the experiments were a joint effort of Dr. Sarah Fowler and the author of this thesis. I would like to thank her for her ideas and time in this collaborative effort.

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Plant Cold Acclimation

Introduction

Plants unlike animals are unable to remove themselves from environmental extremes that they may encounter, including both abiotic and biotic stimuli. To cope with these stimuli plants have evolved a number of complex mechanisms to sense and respond to environmental changes. Often times in the environment a plant has to respond to a complex array of these stimuli including both biotic and abiotic stresses. Low temperature, one such stimuli, limits the growth, distribution, and productivity of plants in the more northern and southern latitudes and at higher elevations. Depending upon their center of origin, plants have different capacities to cope with low temperature. For example, plants that have an origin from tropical and sub-tropical climates may be sensitive to temperatures as low as 15°C (Taylor et al., 1974; Kratsch and Wise, 2000). In contrast, plants from a more temperate climate have evolved mechanisms to cope with low and sub-zero temperatures (Fowler and Gusta, 1979; Scorza et al., 1983). A fundamental biological question is to understand how plants sense and respond to changes in temperature. Furthermore, understanding this may provide insight into improving crop productivity.

The effects of low temperature on plants

Depending upon the type of plant and its surrounding conditions, plants can incur damage as a result of low temperature due to chilling or freezing. Chilling injury primarily occurs in plants from tropical and sub-tropical origins at temperatures as low as 15°C (Taylor et al., 1974; Kratsch and Wise, 2000). A common feature in chilling sensitive species is damage to the chloroplast at low temperatures. This results in the plant's inability to convert light energy to carbon via photosynthesis (Strand et al., 1999). Consequently, chilling sensitive plants exposed to low temperatures for any extensive period of time may die.

As mentioned previously plants can incur damage due to freezing. As the temperature drops below 0°C , ice formation primarily begins in the xylem vessels of leaves and stems and intercellular spaces as these contain fewer solutes and thus have a higher freezing point than the cytoplasmic (intracellular) solution (Thomashow, 1999). As ice forms in these spaces it lowers the water potential in these locations thus creating a gradient between the intracellular and intercellular fluid. This results in movement of water from the cytoplasm to the extracellular space consequently resulting in cytoplasmic dehydration (Thomashow, 1999). This freezing induced dehydration results in damage to the membranes principally (Steponkus, 1984). In addition, freezing can result in protein denaturation and the production of reactive oxygen species (McKersie and Bowley, 1997; Smirnoff, 1998).

Damage to the membrane due to freezing is a function of the absolute temperature reached and the length of time at the temperature. Furthermore, this damage to membranes is mimicked by the damage induced by dehydration and osmotic stress

(Steponkus, 1984; Steponkus et al., 1993b). Three types of membrane damage may result from freeze induced dehydration, these include expansion-induced lysis, lamellar-to hexagonal-II phase transitions and fracture jump lesions (Steponkus et al., 1993a; Uemura and Steponkus, 1997). In the case of expansion-induced lysis, as water moves out of the cell to compensate for the gradient in the water potential, the cell shrinks in size as endocytic vessels bud off the plasma membrane. After the cell thaws, there is influx of water into the cell which causes expansion and lysis of the cell (Steponkus, 1984; Steponkus and Lynch, 1989). Lamellar-to hexagonal-II phase transitions also result from the movement of water back into the cell after the frozen cell thaws. This occurs when the plasma membrane is brought into close proximity to intracellular membranes during freeze induced dehydration. As they come close together it is no longer energetically favorable for the membranes to remain as lipid bilayers. As a consequence when water returns to the cell after thawing the membranes do not reform into bilayers thus the cell has lost its ability to be selectively permemable (Steponkus, 1984; Uemura et al., 1995).

Reactive oxygen species may also be produced during freezing stress. These molecules are produced due to the inability of the chloroplast to convert light energy to ATP (Smirnoff, 1998). Reactive oxygen species can result in further damage to membranes via lipid peroxidation as well as damage to macromolecules such as DNA and denaturation of proteins (Smirnoff, 1998; Kendall and McKersie, 1989). Furthermore, conformational changes in proteins may result from freezing. Potential loss of enzyme activity of certain proteins may result in further damage to the cell.

Cold Acclimation

To counteract the potential damage caused by freezing, some plants have evolved an adaptive response to survive freezing temperatures through a dynamic process termed cold acclimation. Cold acclimation involves changes in gene expression and metabolism in response to low non-freezing temperatures and results in increased freezing tolerance (Weiser et al., 1970). These associated changes are thought to protect the plant against freezing injury.

Changes in Gene Expression

In 1970, Weiser suggested that cold acclimation required the transcriptional activation of a number of genes that were not normally expressed in non-acclimated plants. This was first shown in spinach in 1985 (Guy et al., 1985) and extended to Arabidopsis in 1988 (Gilmour et al., 1988). Since then a number of other groups have identified changes in gene expression in other plants including barley, wheat, and alfalfa (Crosatti et al., 1996; Houde et al., 1992; Monroy et al., 1993). Some of these proteins encoded by these genes have known enzymatic activities that may be important for freezing tolerance.

For example, alcohol dehydrogenase is induced and may aid in anaerobic metabolism when the plant is encased by ice (Nishida and Murata, 1996). Two other examples are phenylalanine ammonia lyase and chalcone synthase, genes that encode proteins important in flavanoid biosynthesis, compounds that may act as sunscreens or antioxidants (Levya et al., 1995). Furthermore, a number of genes that encodes enzymes that scavenge reactive oxygen species, such as glutathione reductase, ascorbate

peroxidase, and superoxide dismutase, are induced at the transcriptional level in response to low temperature (Bridger et al., 1994; Prasad, 1997; Jahnke et al., 1991; Walker and McKersie, 1993; Seppanen and Fegarstadt, 2000). In addition it has been shown the heat shock proteins (HSP's), proteins that act as molecular chaperones, are induced after a freeze thaw cycle (Li et al., 1999).

In Arabidopisis, the best described cold-regulated genes are the COR (cold-regulated) genes. The COR genes have also been termed LTI (low temperature induced), KIN (cold inducible), RD (responsive to dehydration), and ERD (early dehydration inducible) and all are induced by dehydration, high salinity, and ABA (Nordin et al, 1991; Wang et al., 1994; Welin et al., 1994; Wilhelm and Thomashow, 1993; Yamaguchi-Shinozaki and Shinozaki, 1993). There are four groups of COR genes that have been described to date in Arabidopsis. The COR genes exist in tandem pairs in the genome. Key members of each group are *COR6.6*, *COR15a*, *COR47*, and *COR78* (Thomashow et al., 1997). The COR genes encode proteins that are highly hydrophilic, boiling soluble, and predicted to form amphipathic alpha-helices (Lin and Thomashow 1992; Thomashow, 1993). In addition other COR-like genes were recently identified by Fowler and Thomashow (2002).

The best described COR gene to date is *COR15a*, which has been shown to play a direct role in modifying the behavior of membranes upon freezing. COR15a encodes a polypeptide that is targeted to the chloroplast (Lin and Thomashow, 1992). In protoplasts and chloroplasts of transgenic plants over-expressing COR15a one observes a 1°C to 2°C increase in freezing tolerance, however no phenotype is observed in whole plants (Artus et al., 1996). It was also observed that protoplasts over-expressing COR15a formed

lamellar-to hexagonal II phase membrane transitions at a lower temperature (Steponkus et al., 1998). This data provides direct evidence that COR15a functions to protect membranes from freeze induced dehydration.

Transcripts of all the COR genes increased in response to low temperature within 4 hours (Hajela et al., 1990). In response to other stimuli such as dehydration and salinity they all increased similarly. These results suggested that COR genes may be coordinately regulated at a transcriptional level. Promoter anlalysis of the COR genes led to the identification of a *cis*-acting element, the C-Repeat (CRT) or Dehydration Responsive Element (DRE) (Yamaguchi-Shinozaki and Shinozaki, 1994; Baker et al., 1994). This element confers cold, drought, and salt inducible gene expression to the COR genes in an ABA independent manner (Yamaguchi-Shinozaki and Shinozaki, 1994). This *cis*-acting element has been found to be conserved in the promoters of cold responsive genes from a number of species, including wheat and canola (Jiang et al., 1996; Ouellet et al., 1998).

Identification of this *cis*-acting element led to the identification of the transcription factor(s) that binds to it. The C-repeat Binding Factor (CBF) was identified via a yeast one-hybrid assay using the COR15a CRT/DRE promoter element (Stockinger at al., 1997). Furthermore gel mobility shift assays demonstrated that CBF bound to the CRT/DRE. CBF is part of a small gene family, *CBF1*, *CBF2*, and *CBF3* (Gilmour et al., 1998) (also known as *DREB1a*, *b*, and *c* for dehydration responsive element binding factor; Liu et al., 1998), located on chromosome 4 of Arabidopsis as a direct repeat. The CBF protein contains an acidic activation domain, a DNA binding domain similar to the APETALA2 type, and a putative nuclear localization signal (Stockinger et al., 1997).

Over-expression of CBF1 in transgenic Arabidopsis was found to activate the COR genes which contain the CRT/DRE within their promoters (Jaglo-Ottosen et al., 1998). Transgenic plants over-expressing CBF/DREB1 were found to be freezing tolerant as measured by electrolyte leakage assays and whole plant freeze tests in the absence of a low temperature stimulus (Jaglo-Ottosen et al., 1998). Further work has shown that plants over-expressing CBF/DREB1 are also more tolerant to drought and salinity stresses (Liu et al., 1998).

Gene expression analysis has indicated that *CBF* is induced in response to low temperature and mechanical agitation (Gilmour et al., 1998). The data also indicates that CBF is not induced in response to drought, salinity, or ABA (Liu et al., 1998). This would suggest that there are other CRT/DRE binding factors that are induced in response to drought or salinity.

In addition to the CBF/DREB1 family of transcription factors, another group of transcription factors, DREB2 a and b has been identified that binds to the CRT/DRE (Liu et al., 1998). This group of transcription factors is induced in response to drought and salinity but not low temperature. Over-expression of the DREB2 family of transcription factors does not activate the COR genes. However, a transient assay in Arabidopsis protoplasts indicates that this family of transcription factors can activate CRT/DRE containing genes. The authors of this work concluded that some type of post-translational modification of the protein was required for the protein to bind to the CRT/DRE. The above work has led Shinozaki and colleagues to propose that the CBF regulon of CRT/DRE containing genes is activated by two independent families of transcriptional activators, CBF/DREB1 and DREB2, which act in two separate signal

transduction pathways under low temperature and dehydration respectively (Liu et al., 1998).

Recent work from Zarka et al. (2003) has shown that the cold induction of the *CBF* genes involves multiple ICE (Inducer of CBF Expression) promoter elements. Further work from Chinnusamy et al. (2003) has shown that a mutant, *ice1*, blocks the cold induced expression of *CBF3* but not *CBF1* or *CBF2*. Furthermore they demonstrated that the *ice1* mutation decreases the expression of a number of other cold regulated genes.

Recent transcript profiling experiments have shown that the CBF cold response pathway is only a subset of all the genes up-regulated in response to low temperature (Fowler and Thomashow, 2002; Seki et al., 2001). Fowler and Thomashow (2002) proposed that there may be a number of other parallel cold response pathways in addition to CBF. These pathways may be important for the induction of other cold-regulated genes that may play a role in the development of freezing tolerance.

Natural variation in freezing tolerance has recently been reported in two Arabidopsis ecotypes, Landsberg erecta (Le) and Cape Verde Islands (Cvi). This variation led to the identification of quantitative trait loci, one of them being CBF (Martinez-Zapater et al., 2002). This data provides further evidence of the relative importance of CBF to cold acclimation and the development of freezing tolerance.

Changes in Small Molecules and Membranes

Many metabolic changes have been described that are associated with cold acclimation. In response to low temperature, plants accumulate a number of small

molecules known as compatible solutes (Smirnoff, 1998). Examples of compatible solutes include soluble sugars such as glucose, fructose, sucrose, and raffinose as well as amino acids such as proline (Bohnert and Sheveleva, 1998; Strauss and Hauser, 1986). Compatible solutes such as these are thought to act as osmo-protectants by maintaining the turgor of the plant cell during freeze induced dehydration by lowering the osmotic potential of the cytoplasm. In addition they are thought to be cyroprotective by protecting proteins from denaturation and stabilizing membranes (Smirnoff, 1998; Steponkus, 1984; Strauss and Hauser, 1986). Interestingly, many of these metabolic changes are associated with other osmotic stresses such as dehydration and salinity (Steponkus, 1984). These changes may be a result of an adaptive process to protect the plants against freezing injury or the result of metabolic disturbances caused by low temperature (Bohnert and Sheveleva, 1998). The importance of these metabolic changes to freezing tolerance and other osmotic stresses has been implicated by the following studies. First, over-expression of the transcription factor CBF3 results in plants that have increased tolerance to freezing, dehydration, and salinity which is correlated with increased levels of soluble sugars and proline (Liu et al., 1998; Gilmour et al., 2000). Second, antisense plants of the proline dehydrogenase gene, a gene that encodes a protein responsible for proline breakdown, show elevated levels of proline and show increased freezing and dehydration tolerance (Nanjo et al., 1999). Third, over-expression of galactinol synthase, a gene that encodes a protein responsible for galactinol and raffinose biosynthesis, results in increased tolerance to dehydration (Taji et al., 2002). Although these experiments show only correlations; they do not exclude the possibility of other changes that may be responsible for the observed phenotypes.

In addition to these changes, phospholipid ratios and their saturation level are modified in response to low temperature (Uemura et al., 1995; Uemura and Steponkus, 1997). These changes are thought to increase the stability of membranes. For example, changes in the plasma membrane in acclimated plants result in the production of exocytic extrusions rather than the more damaging endocytic vesicles that form under freezing conditions in non-acclimated plants (Steponkus et al., 1988; Uemura et al., 1995). In addition, changes in the chloroplast membranes reduce the propensity to form hexagonal II phase transitions (Uemura and Steponkus, 1997). These changes in membrane composition are thought to increase the cyrostability of membranes, thus protecting the plant against freeze induced dehydration damage (Uemura et al., 1995; Uemura and Steponkus, 1997).

Role of Abscisic Acid (ABA) in cold acclimation

ABA has been implicated in a number of stress responses. ABA levels have been shown to transiently increase in response to low temperature however they decrease back to wild-type levels within two days despite the maintenance of the acclimated state (Lang and Palva, 1992; Gilmour et al., 1988). Furthermore application of ABA in Arabidopsis and *Solanum commersonii* has been shown to promote cold acclimation. In addition ABA levels have been observed to change transiently in response to low temperature in *S. commersonii*, a plant that can cold acclimate, but not in *S. tuberosum*, a plant that does not cold acclimate. This has led to the hypothesis that ABA is required for cold acclimation. Moreover, ABA induces the transcription of many genes that are also

induced by low temperature stress, including those containing the CRT/DRE motif. These results have led to the hypothesis that ABA is required for cold acclimation.

To test this hypothesis, mutants in Arabidopsis affected in ABA biosynthesis and mutants altered in ABA signaling were examined for the ability to cold acclimate. It was found that plants having defects in either the biosynthesis of ABA (*aba1*; *ABA1* encodes a zeoxanthin epoxidase) or the perception of ABA (*abi1*; *ABI1* encodes a protein phosphatase 2C) (Koorneef et al., 1998) were less freezing tolerant than wild type plants after cold acclimation, providing evidence that ABA is required for cold acclimation (Gilmour and Thomashow, 1991; Heino et al., 1990). Interestingly, in both classes of mutants, *aba* and *abi*, COR gene expression does not differ from that of wild-type in response to low temperature (Gilmour and Thomashow, 1991). This observation is of particular significance because the COR genes are responsive to both ABA and low temperature. This has led to the hypothesis that activation of the COR genes occurs independently of ABA in response to low temperature via the CRT/DRE *cis*-acting element (Gilmour and Thomashow, 1991; Yamaguchi-Shinozaki and Shinozaki, 1994). So why are these mutants in ABA biosynthesis and signaling less freezing tolerant? It may be due to lack of ABA or the ability to perceive it or it may be simply due to the fact that these mutants exhibit pleotropic phenotypes when grown at warm temperatures. For example they are wilty and are generally less robust even in the absence of low temperature (Koorneef et al., 1998).

Recent work has demonstrated that changes in the expression of genes involved perception and propagation of the ABA signal can influence cold acclimation (Tahtiharju and Palva, 2001; Tamminen et al., 2001). However, these observations did not

demonstrate the requirement of ABA for cold acclimation. They simply demonstrate that alteration of ABA signaling and application of ABA will substitute for the stimulus of low temperature and induce cold acclimation. These experiments show that cross talk exists between ABA signaling and cold signaling pathways.

The Role of Calcium

One of the initial responses of plants to low temperature is a rapid transient elevation of the cytoplasmic calcium levels. This response has been observed in alfalfa and Arabidopsis (Knight et al., 1991; Monroy and Dhindsa, 1995). An elevation of calcium levels has been seen to occur in response to a rapid change in temperature (Knight et al., 1991, Knight et al., 1996) but also in response to slow changes in temperature (Plieth at al., 1999). The majority of the calcium comes from extracellular stores (Knight et al., 1996, Monroy and Dhindsa, 1995); however there is some evidence for inositol 1,4,5 trisphosphate-mediated calcium release from intracellular stores (Knight et al., 1996). This rapid transient change in calcium is required for the cold regulated gene expression of *KIN1* and *KIN2*, two CRT/DRE and ABRE containing genes (Knight et al., 1996, Tahtiharju et al., 1997). Furthermore these observations were extended to demonstrate that blocking the calcium elevation by calcium channel blockers inhibits the process of cold acclimation (Monroy and Dhindsa, 1995). In summary, a transient increase in calcium levels is required for cold acclimation and the induction of some cold regulated genes.

This elevation in calcium is hypothesized to affect the activity of kinases and phosphatases that later influence gene expression. It has been shown that inhibitors of

calmodulin and calcium dependent protein kinases effect the expression of *KIN1* and *KIN2* and the development of freezing tolerance (Tahtiharju et al., 1997). It has been demonstrated that a number of proteins are phoshorylated in response to low temperature and that this phosphorylation is dependent upon the rapid influx of calcium and the activity of calcium dependent protein kinases and calmodulin (Tahtiharju et al., 1997). Other results suggest that calcium dependent phosphorylation of proteins is important for cold acclimation and that the activity of protein phosphatases may important to cold acclimation (Monroy and Dhindsa, 1998).

Recent work by Dhindsa and colleagues (2000) has provided some evidence as to how low temperature triggers this calcium influx. A decrease in temperature is known to alter the fluidity of the membrane (Orvar et al., 2000). This change in membrane fluidity is hypothesized to change the activity of ion channels which could result in the elevation of calcium levels. Dhindsa and colleagues (2000) showed that a membrane fluidizer, DMSO, prevented the induction of *cas30* and *BN115* in response to low temperature and the development of freezing tolerance. In contrast, they showed that membrane rigidification resulted in the induction of *cas30* and *BN115* and an increase in freezing tolerance without a low temperature stimulus. In addition, they demonstrated membrane rigidification is followed by a reorganization of the cytoskeleton, specifically actin microfilaments. Following this, there is a rapid elevation of calcium and subsequent change in gene expression. Recent work from other groups has shown further that cortical microtubular rearrangement occurs in response to low temperature (Abdrakhamanova et al., 2003).

Influence of the circadian clock and light

Previous work has shown that the circadian clock can be entrained by low temperature (Kreps and Simon., 1997). Furthermore, it has been shown in some plants that there are rhythms in chilling tolerance that peak in the evening that are under the control of the circadian clock (Kreps and Simon., 1997). Recently it was shown by Harmer at al. (2000) that *CBF3/DREB1a* transcript levels cycle in a circadian fashion. *CBF3/DREB1a* transcript levels peaked 4 hours after dawn. This peak was followed about 4-8 hours later by peaks in the transcript levels of several targets of CBF including some of the COR genes. These results were postulated as a possible mechanism to explain the circadian rhythm in chilling tolerance.

Light is known to be one of the many inputs into the circadian clock. Recently two different groups have shown how light affects *CBF* expression in the absence of a low temperature stimulus and how light mediates the induction of the CBF cold response pathway in response to low temperature. First, it was shown that *CBF* is induced in response to far red light in the absence of a low temperature stimulus and that this induction was mediated via the PHYA signaling pathway (Tepperman et al., 2001). Second, it was shown by Kim et al. (2002) that light was required for the maximum induction of *CBF*. In addition they showed that a tetramer of CRT/DRE driving the GUS reporter gene required light to be induced in response to low temperature. Furthermore they showed that PHYB was required for induction of this reporter gene in the light. These results suggest that light is required for the induction of the COR genes and that its induction is mediated by PHYB.

Freezing tolerance Genes by Mutational Analysis

To further understand how plants cold acclimate and to identify critical components important to the development of freezing tolerance a mutational approach has been taken. One approach was to isolate mutants that were unable to cold acclimate or mutants that exhibited a constitutively cold acclimated phenotype. Another approach was to identify mutants with altered expression of the COR genes or the CBF genes.

Warren et al. (1996) developed a screen to identify mutants unable to cold acclimate which would be sensitive to freezing (SFR). They identified 7 mutants that were freezing sensitive. The freezing sensitivity in each of these mutants varied significantly after exposure to freezing temperatures, for example in *sfr1* only the young leaves were affected while in *sfr2*, *sfr4*, *sfr5*, and *sfr7* all leaves were affected to various degrees. The plasma membranes stability was also affected in all of the mutants as indicated by the electrolyte leakage assays. Interestingly the *sfr2* mutant sustains significant damage to leaf tissue as seen in the whole plant freeze test but only minor affects are observed in the electrolyte leakage assay. This observation suggests that primary site of freezing damage in the *sfr2* mutant is something other than the plasma membrane.

Isolation of the SFR genes and analysis of their function should provide significant understanding of how plants cold acclimate and how they change physiologically in response to cold acclimation. For example *sfr3*, *sfr4*, *sfr6*, and *sfr7* mutations reduced or eliminated the accumulation of anthocyanin in response to cold acclimation. In addition the *sfr4* mutation prevented increases of sucrose and glucose normally observed in wild-type plants during cold acclimation and affected fatty acid

composition in response to cold acclimation. Other mutations such as *sfr1, sfr2*, and *sfr5* did not affect anthocyanin levels, sugar accumulation, or changes in fatty acid composition suggesting they may affect cold acclimation in more specific ways yet to be identified (Warren et al., 1996).

Another mutation, *sfr6*, was found to suppress the low temperature induction of genes containing the CRT/DRE *cis*-acting element (Knight et al., 1999). The induction of the COR genes in response to ABA and mannitol was also impaired to a lesser extent in the sfr6 mutant. The failure to induce these genes was not due to loss of CBF expression, the trans-acting factor that binds to this *cis*-element. *CBF1*, *CBF2*, and *CBF3* were induced to wild-type levels in cold acclimated sfr6 mutants. The observation that the suppression of CRT/DRE containing genes in response to low temperature, mannitol, and ABA suggests the possibility of cross talk between ABA independent and ABA dependent pathways. It was also determined that the suppression of these genes was not due to altered calcium signaling. Cold induced cytolosic-free calcium was found to be indistinguishable from wild-type in the *sfr6* mutation. Interestingly the *sfr6* mutation also has pleiotropic affects on pigmentation and fertility in warm-grown plants. These observations suggest that the protein encoded by the SFR6 gene is not only required for the cold induced CBF induction of the COR genes but is also required for induction of genes required for normal plant growth and development. Possibly the SFR6 protein may act in the formation of transcriptional activation complex with CBF and other transcription factors.

Xin and Browse (1998) developed a screen to identify mutants that displayed a constitutive freezing tolerant phenotype; plants that were more freezing tolerant than

wild-type without cold acclimation. This result led to the identification of a single mutant, *eskimo1*. Non-acclimated *esk1* plants were more freezing tolerant than wild-type; in addition, *esk1* mutants acclimated to a lower temperature when compared to wild-type.

The mutated gene responsible for the increase in freezing tolerance has not yet been identified. However, some initial experiments provide some explanation for the observed increase in freezing tolerance. *esk1* plants accumulate more free proline than non-acclimated wild-type plants providing more correlative evidence of the significant role of this compatible solute. Interestingly, cold acclimation of *esk1* plants does not result in the subsequent increase in free proline as wild-type plants when they are cold acclimated. In addition, transcript levels of the committed step in proline biosynthesis, P5CS, are elevated in non-acclimated *esk1* plants. Furthermore, the transcript levels for POX1, the gene that encodes for proline degradation, are not changed. This large increase in proline likely explains the observed increase in freezing tolerance as proline has been shown to be a cyroprotectant in vitro (Rudolph and Crowe, 1995). In addition, soluble sugars are elevated in the *esk1*. Another significant observation was *esk1* mutants do not have altered transcript levels of the COR genes in non-acclimated plants or in cold acclimated plants. This observation led Xin and Browse (1998) to speculate the existence of multiple parallel pathways that are required for cold acclimation. This data would suggest that activation of one of these pathways can significantly alter the freezing tolerance of plants without activation of the other independent pathways. The *esk1* mutation is recessive suggesting that ESK1 normally acts as a negative regulator of proline biosynthesis.

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Chapter 2

Transcription Factor CBF4 is a Regulator of Drought Adaptation in Arabidopsis

Summary

In plants, low temperature and dehydration activate a set of genes containing CRT/DRE elements in their promoter. Previously it has been shown that the Arabidopsis CBF/DREB1 transcription activators are critical regulators of gene expression in the signal transduction of cold acclimation. Here we report the isolation of an apparent paralog of the CBF/DREB1 proteins, CBF4, that plays the equivalent role during drought adaptation. In contrast to the three already identified CBF/DREB1 parologs, which are induced under cold stress, *CBF4* gene expression is up-regulated by drought stress, but not by low temperature. Overexpression of CBF4 in transgenic Arabidopsis plants results in the activation of CRT/DRE element containing downstream genes that are involved in cold acclimation and drought adaptation. As a result, the transgenic plants are more tolerant to freezing and drought stress. Because of the physiological similarity between freezing and drought stress, and the sequence and structural similarity of the CBF/DREB1 and the CBF4 proteins, we propose that the plant's response to cold and drought evolved from a common CBF like transcription factor, first through gene duplication and then through promoter evolution.

Introduction

Many plants increase their tolerance to freezing after exposure to low non-freezing temperatures – a phenomenon known as cold acclimation (Hughes and Dunn, 1996, Thomashow, 1998). The major component of this acquired freezing tolerance is the tolerance to dehydration stress. Freeze induced-dehydration is caused by extracellular ice formation during the freezing process. The presence of ice lowers the water potential extracellularly and causes water to flow out of cells (Pearce, 1999). Thus, a major cause of freezing damage is freeze-induced dehydration (Thomashow, 1998; Steponkus and Webb, 1992). Since a plant's ability to survive freeze-induced dehydration is related to its adaptation to drought, it is not surprising that plants respond to low temperature and drought very similarly at the molecular level (Shinozaki and Yamaguchi-Shinozaki, 2000). Many genes, such as RD (responsive to dehydration), ERD (early responsive to dehydration), COR (cold-regulated), LTI (low-temperature induced), and KIN (cold-inducible), are induced by both low temperature and drought stress (Pearce, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000). The similarity of cold and drought stresses is further demonstrated by experiments showing that mild drought stress can result in increased freezing tolerance in plants (Guy et al., 1992; Siminovitch and Cloutier, 1983, Clavitier and Siminovitch, 1982).

Recently, a major transcriptional regulatory system that controls abscisic acid (ABA) independent gene expression in response to low temperature has been identified (Stockinger et al., 1997; Liu et al., 1998). The system is based on the CRT/DRE (C-repeat or dehydration responsive element) *cis*-acting element and the *trans*-acting DNA binding protein CBF/DREB1 (C-repeat binding factor or DRE binding protein). There are

three CBF/DREB1 genes present in the Arabidopsis genome arranged in a tandem array within a region of 8.7 kb (Gilmour et al., 1998; Medina et al., 1999). Their expression is induced by low temperature, and they, in turn, activate the expression of many low temperature responsive genes (Seki et al., 2001). When overexpressed constitutively in Arabidopsis plants, they induce the expression of downstream genes under non-stress conditions, and confer freezing, drought, and salt tolerance to the transgenic plants (Kasuga, et al. 1999, Jaglo-Ottosen et al, 1998, Gilmour et al., 2000). Therefore, this class of genes represents a critical component in the signal transduction of cold acclimation.

Because the CRT/DRE element is sufficient for drought inducible gene expression (Yamaguchi-Shinozaki and Shinozaki, 1994), it strongly suggests the existence of drought inducible transcription factors that bind to the element. In an earlier study, two transcription factors, DREB2a and DREB2b, were identified based on their ability to bind the CRT/DRE element in vitro and in yeast (Liu et al., 1998). Because they are induced by drought stress and are able to induce the expression of genes that contain the CRT/DRE *cis*-acting element in protoplast transient assays, they were good candidates to be involved in drought signal transduction. Interestingly, overexpression of the DREB2 cDNA in transgenic plants only caused weak induction of the downstream genes and did not result in obvious phenotypes (Liu et al., 1998). It is proposed that translational modifications are necessary for the activity of those proteins in transgenic plants (Shinozaki and Yamaguchi-Shinozaki, 2000), but this hypothesis remains to be demonstrated experimentally.

In this study, we report the isolation of CBF4, a gene coding for a protein that is the closest homologue of CBF/DREB1 proteins in Arabidopsis. The expression of *CBF4* is induced rapidly during drought stress and by ABA treatment, but not by cold. The overexpression of CBF4 under the constitutive 35S CaMV promoter resulted in the expression of cold and drought induced genes under non-stress conditions. The transgenic plants are also more tolerant to freezing and drought conditions. Thus, we suggest that CBF4 plays a role in the signal transduction of drought adaptation in Arabidopsis plants. Because of the high sequence similarity between CBF4 and the CBF/DREB1 proteins, especially in the DNA binding domain, downstream gene activation is presumably through the binding of CRT/DRE element. We propose that the signal transduction of cold acclimation and drought adaptation in pathways involving the CRT/DRE element evolved from a common CBF/DREB1 locus first through gene duplication and then through the evolution of their promoters.

Results

CBF4 is the closest homolog of CBF/DREB1 proteins.

As a part of a functional genomics program on Arabidopsis transcription factors, one protein was recognized as the AP2/ERF protein (At5g51990) most closely related to the three previously described Arabidopsis CBF/DREB1 proteins (CBF1/DREB1b: At4g25490; CBF2/DREB1c: At4g25470; CBF3/DREB1a: At4g25480) (Stockinger et al., 1997; Gilmour et al., 1998). We designated the gene coding for the protein CBF4. The CBF4 open reading frame was initially detected in the sequence of P1 clone MSG15 (genbank accession # AB015478), and it was shown by RACE to correspond to an

intronless and expressed gene. Phylogenetic relationships between CBF1-4 and At1g63030 and At1g12610, the two most closely related genes to CBF1-4 within the Arabidopsis AP2/ERF family are shown in Figure 2.1A. CBF4 is the only other member of the AP2/ERF transcription factor family for which substantial sequence similarity with CBF1-3 extends beyond the conserved AP2 domain (Figure 2.1B). CBF4 is 224 amino acids in length, shares 63% overall amino acid sequence identity with the three CBF/DREB1 proteins, and 91 to 94% identity within the AP2/ERF DNA binding domain (Figure 2.1B). It has been noted recently that all CBF/DREB1 proteins share common signature motifs that bracket the AP2 domain, and those motifs are found in CBF-like proteins that are conserved across species (Jaglo-Ottosen et al., 2001). Both signature motifs are present in the CBF4 protein (Figure 2.1B). In the phylogenetic analysis of the complete Arabidopsis AP2/ERF gene family, which consists of at least 144 members, At1g71450 is next to, but falls outside of, the clade defined by the other six proteins, and therefore it is included in the multiple sequence alignment as a control (Figure 2.1B).

CBF4 overexpression results in growth retardation and activation of COR genes.

The high degree of sequence similarity between CBF4 and CBF1, 2 and 3, suggested that the proteins were functional homologs. To test this hypothesis, the CBF4 gene was cloned from cDNA, and constitutively overexpressed under the control of the cauliflower mosaic virus 35S promoter in transgenic Arabidopsis plants. The effects of CBF4 overexpression on plant growth, COR gene expression and stress tolerance were tested using the transgenic plants (compared to the published sequence, At5g51990, the clone used for analysis contained a single point mutation that changed amino acid residue

Figure 2.1

Phylogenetic analysis and sequence comparison of CBF4.

(A) Phylogenetic tree showing the relationships between CBF4, CBF1-3, At1g63030, and At1g12610. The neighbor-joining tree was based on an alignment of the complete protein sequences. Bootstrap values are shown on branches. At1g63030 and At1g12610 are the two most closely related genes to CBF1-4 within the Arabidopsis AP2/ERF family. Addition of other AP2/ERF family members does not change the phylogenetic tree (data not shown).

(B) Sequence comparison of the proteins CBF4, CBF1-3, At1g63030, At1g12610, and Atg71450. At1g63030 and At1g12610 are the two most closely related genes to CBF1-4 within the Arabidopsis AP2/ERF family. In the phylogenetic analysis of the complete gene family, Atg71450 is next to, but falls outside of, the clade defined by the other six proteins. The predicted AP2 domains and the two signature regions, I and II, previously noted for the CBF1-3 proteins (Jaglo et al., 2001) are also shown.

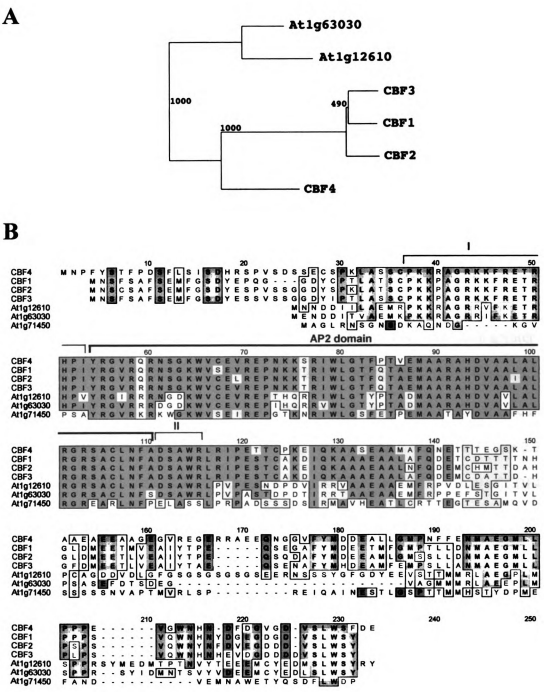

Figure 2.1

Phylogenetic analysis and sequence comparison of CBF4.

111 from D to G). Thirty independent transgenic lines were identified by kanamycin selection (the selectable marker carried in the transformation vector). Similar to earlier observations of CBF3/DREB1a overexpression using the 35S promoter (Kasuga et al., 1999, Gilmour et al., 2000), the 35S::CBF4 plants showed retarded growth compared to the wild type controls (Figure 2.2A), had shorter petioles and darker green leaves, and the time to flowering was significantly delayed (data not shown). For a detailed characterization, three transgenic lines (L10, L48 and L2) representing the full range of different sizes from minor phenotypic differences (L10) to severe growth retardation (L2) were chosen. Northern analysis indicated that under both normal and cold-acclimated conditions, the transcript levels for CBF4 in the transgenic plants were much greater than those observed for the control plants (wild-type and empty vector control, Figure 2.2B). The transcript level for CBF4 did not change significantly upon cold-acclimation either in the wild type or in the transgenic plants. The degree of growth retardation (Figure 2.2A) correlated with the level of CBF4 expression (Figure 2.2b): the highest level of CBF4 expression caused the most severe growth phenotype.

In response to dehydration stress such as cold and drought, wild-type Arabidopsis plants induce the expression of a large number of genes including the COR genes (Seki et al., 2001). It has been shown previously that overexpression of CBF1/DREB1b (Jaglo-Ottosen et al., 1998) or CBF3/DREB1a (Kasuga et al., 1999, Gilmour et al., 2000, Seki et al. 2001) is sufficient to induce the expression of the downstream CRT/DRE element containing COR target genes. To investigate whether the CBF4 transcription factor could also activate the COR genes, expression levels for *COR15a* and *COR78*, known downstream target genes of CBF/DREB1 proteins, were analyzed and shown in figure

2b. In the control plants (wild type and empty vector control), the *COR15a* and *COR78* transcript levels were low under normal growth conditions and increased after cold-acclimation. Constitutive expression of the *CBF4* gene resulted in the expression of both *COR15a* and *COR78a* without a low temperature or drought stimulus (Figure 2.2B). Compared to the controls, the *COR15a* and *COR78a* transcript levels were higher in all three transgenic lines, and only increased in response to cold in the weakest overexpressor, line L10.

To ensure that the induction of the COR genes was due to CBF4 overexpression rather than the expression of other *CBF/DREB1* genes, *CBF1/DREB1b* gene expression was analyzed as well (the probe used cross-reacts with CBF2 and CBF3). Figure 2.2B illustrates that the *CBF1/DREB1b* message was low under normal conditions in all plants, and showed a very similar increase in response to cold in both control and transgenic plants. To further demonstrate the direct involvement of CBF4 in the activation of the COR genes, we used a transient assay developed by Yang et al. (2000) to test CBF4's ability to induce the expression of a *COR78* promoter::GUS construct (Horvath et al., 1993). The GUS reporter gene under *COR78* promoter was induced 11.2±2.7 fold by CBF4 and 12.3±1.0 fold by CBF3 in this assay (induction level was calculated relative to the empty vector control; data presented were averages of at least eight independent tests). As a control, At4g36900, another AP2 domain transcription factor, was not able to induce the COR78a promoter (0.4±0.1 fold). Taken together, we concluded that the COR gene induction in transgenic plants was a direct consequence of CBF4 expression, and similar to the other CBF/DREB1 genes, CBF4 is a regulator of COR gene expression.

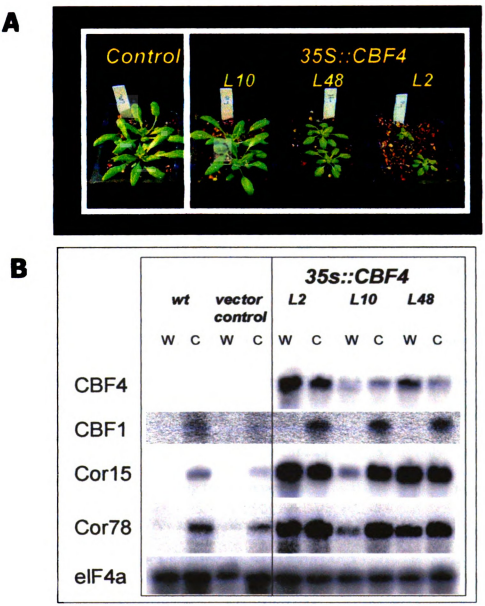

Figure 2.2

Growth characteristics of and transcript levels in CBF4 overexpressing transgenic plants.

(A) Control plants (transformed with empty vector) and CBF4 overexpressing lines L10, L48, and L2 after 4 weeks growth at 22°C.

(B) Northern analysis of total RNA. Ten µg total RNA prepared from wild type and empty vector control Arabidopsis plants and from CBF4 overexpressing lines L10, L48, and L2 were blotted and probed with the indicated probes. Plants were either grown at 22°C for 14-15 days (W) or at 22°C for 12-14 days and then cold treated at 4°C for 7 days (C). *eIF4a* is a constitutively expressed gene used as a loading control (Metz et al., 1992).

CBF4 overexpression results in plants that are more freezing and drought tolerant.

Overexpression of CBF1/DREB1b (Jaglo-Ottosen et al., 1998) or CBF3/DREB1a (Liu et al., 1998, Kasuga et al., 1999, Gilmour et al., 2000) has been reported to increase the freezing, drought and salt stress tolerance of non-acclimated transgenic plants. To further demonstrate the functionality of the CBF4 gene, we examined the degree of freezing and drought tolerance of the transgenic plants. In a whole plant freezing assay, two week old plants grown on petri dishes, with or without cold acclimation for four days at 4°C, were frozen for 20 hours at -15°C (cold-acclimated plants) or -10°C (non-acclimated plants), respectively. Plant survival was scored after two days of recovery under normal growth conditions, and representative results are shown in Figure 2.3A. The constitutive overexpression of the CBF4 gene resulted in an increase in freezing tolerance of the transgenic plants under both cold-acclimating and non-acclimating conditions (Figure 2.3A, Table 2.1). Under non-acclimating conditions only 1% of the wild type plants survived the freezing test compared to 52 to 100% for the different transgenic lines (Table 2.1). Very similar results were obtained after cold-acclimation with a 2% survival rate for the wild type, and 54 to 89% for the different transgenic lines (Table 2.1).

To quantify the degree of added freezing protection, electrolyte leakage experiments were done. Under non-acclimating conditions (Figure 2.4A), the EL_{50} (temperature that causes 50% leakage) value for the control was -2.6°C compared to -5.6°C (L10) and up to -13.3°C (L2) for the 35S::CBF4 plants. The level of freezing tolerance correlated with the level of CBF4 overexpression. Cold-acclimation (Figure 2.4B) further increased the level of freezing tolerance to -7.9°C for the control, and -

9.5°C (L10) and up to -18.7°C (L2) for the transgenic CBF4 plants. Therefore, without cold acclimation, high level overexpression of CBF4 was sufficient to result in at least the same level of freezing tolerance as cold acclimated control plants. After cold acclimation the level of freezing tolerance increased even further in the 35S::CBF4 plants. This result is similar to what has been reported for the overexpression of CBF1/DREB1b (Jaglo-Ottosen et al., 1998) and CBF3/DREB1a genes (Kasuga et al., 1999, Gilmour et al., 2000).

To assay if the transgenic plants are more drought tolerant than the wild type controls, 14 day old plants grown on soil were held without water for nine days before the plants were watered again (Figure 2.3B). While only 2% of the wild type plants survived this treatment, the survival rate for the 35S::CBF4 plants was much higher with 45% for L10 and 87% for L48 (Table 2.1; L2 was not included in this test). The increased survival rate four days after re-watering illustrates that the overexpression of CBF4 resulted in transgenic plants that are significantly more drought tolerant than the wild type controls. Again, the level of drought tolerance correlated with the level of CBF4 overexpression, similar to what has been previously reported for CBF3/DREB1a (Kasuga et al., 1999). From these and the other results described above, we concluded that CBF4 is a fully functional fourth member of the CBF/DREB1 family.

CBF4 is induced by drought and ABA.

The *CBF/DREB1* genes are induced by cold, but not by drought or ABA (Shinwari et al., 1998, Medina et al, 1999). To understand the role CBF4 might play in the signal transduction of dehydration stress response, we studied its gene expression in response to low temperature, drought, and the exogenous application of ABA. RNA from

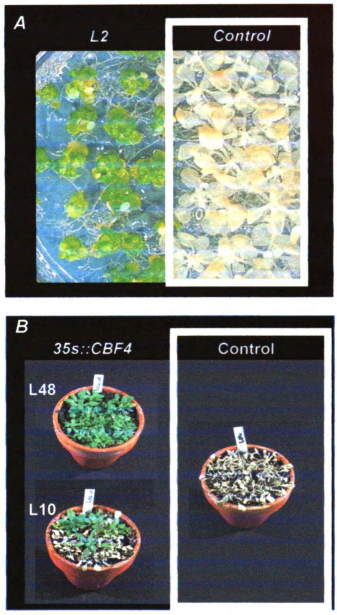

Figure 2.3

Effects of freezing and drought stress on transgenic plants over-expressing CBF4.

(A) Transgenic plants from line L2 and empty vector controls were grown side by side on petri dishes for two weeks, then frozen at –10°C for 20 hours. Photographs were taken two days after transfer to 22°C.

(B) Plants were grown for two weeks with normal watering, withheld from water for 9 days, and re-watered for 4 days before photographs were taken. Control and transgenic plants are shown under the same conditions.

Table 2.1.

Survival rates of transgenic plants under different stress conditions.

	Survival[d]	Total[e]	% Survival[f]
(A) Freezing tolerance (non-acclimated) [a]			
Control	1	169	1
L10	44	85	52
L48	29	35	83
L2	44	44	100
(B) Freezing tolerance (cold acclimated)[b]			
Control	4	220	2
L10	50	91	55
L48	15	28	54
L2	73	82	89
(C) Drought tolerance[c]			
Control	3	181	2
L10	44	98	45
L48	123	141	87

(a) Two weeks old plants grown on petri dishes at 22°C and then frozen at –10°C for 20 hours

(b) Two weeks old plants grown on petri dishes at 22°C, transferred to 4°C for 4 days, and then frozen at -15°C for 20 hours

(c) Two weeks old soil grown plants withheld water for 9 days, re watered, and scored four days later. Plants were considered dead if all the leaves were brown and there is no re-growth 4 days after re-watering. Water loss during the drought period was similar for all pots independent of the plant size (data not shown)

(d) Number of plants that survived

(e) Total number of plants used in each assay

(f) Percentage of plants that survived

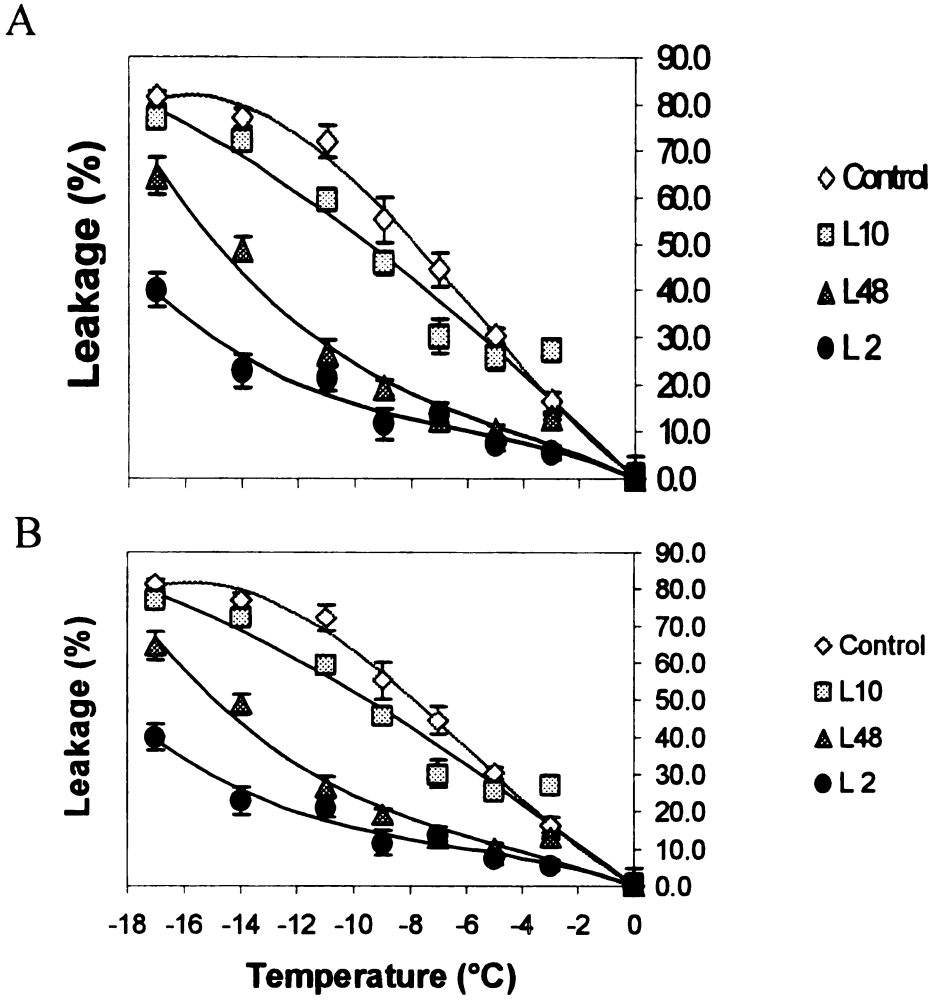

Figure 2.4

Quantification of freezing tolerance of transgenic plants overexpressing CBF4 gene. Using the electrolyte leakage assay, freezing tolerance was measured in plants grown on petri dishes for two weeks without (A) and with cold acclimation at 4°C for 7 days (B). Model curves fitting up to third-order linear polynomial trends were determined for each electrolyte leakage experiment. Based on those EL_{50} (temperature causing 50% ion leakage) values were calculated for non-acclimated (control: -2.6°C; L10: -5.6 °C; L48: -8.6 °C; L2: -13.3 °C) and cold-acclimated conditions (control: -7.9 °C; L10: -9.5 °C; L48: -14.9 °C; L2: -18.7 °C).

plants subjected to low temperature, drought, or the exogenous application of ABA were blotted and hybridized with probes for *CBF4, COR15a*, and *elF4a* (this gene is not responsive to cold, drought or ABA). Figure 5a showed that, under our experimental conditions, *CBF4* was not responsive to cold. However, transcripts for *CBF4* did accumulate in response to water deficit (Fig. 5b) and exogenous application of ABA (Fig. 5c). To demonstrate that the induction of *CBF4* expression is ABA dependent, *CBF4* gene expression was analyzed in the ABA deficient mutant *aba1-1* (Koornneef et al., 1982). In *aba1-1*, the drought induction of CBF4 expression is dramatically reduced (Fig. 5b), indicating that ABA biosynthesis is required for the proper drought induced induction of CBF4 expression.

Discussion

In this study, we report the isolation of CBF4 as part of a functional genomics research program on transcription factors. The gene encodes a protein that is the closest homologue to the CBF1,2,3/DREB1abc transcriptional activators in Arabidopsis. Although the isolated clone contains a single amino acid change from the published genomic sequence, it appears that this change did not result in a change of its function. The sequence alignment presented in figure 1 shows that two signature motifs which are conserved among CBF genes across different species (Jaglo et al., 2001) are also present in CBF4. Overexpression of CBF4 using the constitutive CaMV 35S promoter resulted in constitutive expression of COR15a and COR78a, both of which are cold and drought inducible and known target genes for CBF1, 2 and 3 (Gilmour et al., 1998, Kasuga et al.,

Figure 2.5.

Gene expression analysis of *CBF4*

Total RNA was prepared from plate grown Arabidopsis plants subjected to (A) low temperature (4°C), (B) dehydration, and (C) ABA blotted and probed with indicated probes. (A) RNA was isolated from plants that were placed in a cold room set at 4°C for the indicated amount of time. (B) RNA was isolated from both wild type and aba1-1 plants placed over desiccant for the indicated amount of time. (C) RNA was isolated from plants placed in Gamborgs B5 liquid with 100 μm ABA for the indicated amount of time. For controls in (B) and (C), the plants were transferred to Gamborgs B5 liquid (see materials and methods).

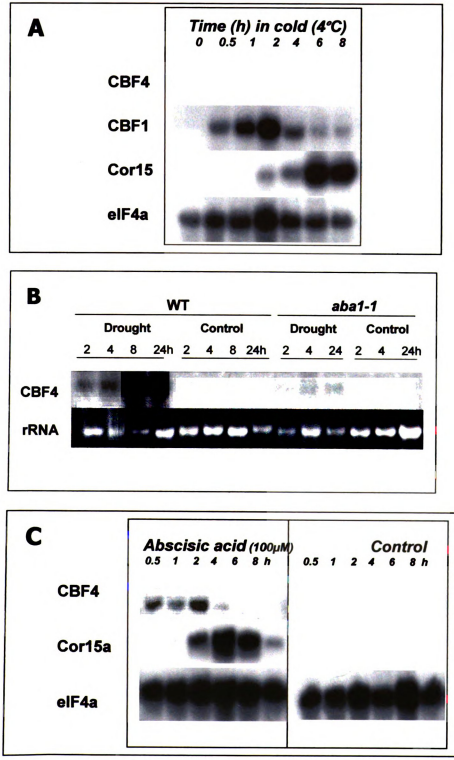

Figure 2.5

Gene expression analysis of *CBF4*.

1999). As a result, the transgenic plants are also more tolerant to freezing and drought stress. All of the above phenotypes have been observed for the constitutive overexpression of CBF1 (Jaglo-Ottosen et al., 1998) and CBF3/DREB1a (Liu et al., 1998, Kasuga et al., 1999; Gilmour et al, 2000), indicating that CBF4 is in fact a new and functional member of the CBF/DREB1 family. Interestingly, the level of dehydration tolerance that can be achieved by CBF4 overexpression is very similar to that achieved by over-expressing other CBF/DREB1 genes, suggesting that all CBF/DREB1 genes induce the expression of a common set of CRT/DRE target genes.

Unlike that of the other CBF/DREB1 transcription factors (Medina et al., 1999; Gilmour et al., 1998; Shinwari et al., 1998), the expression of *CBF4* does not change in response to cold, but is induced by drought stress and ABA treatment (Fig. 5). In an earlier study, two cDNAs encoding AP2 domain transcription factors, DREB2a,b were identified by a yeast one hybrid screen (Liu et al., 1998). Both bind to the CRT/DRE element in vitro, and activate expression from the COR78a/RD29a promoter in a transient protoplast assay (Liu et al., 1998). The drought, and salt responsive expression pattern of the *DREB2* genes suggests a role in the activation of the CRT/DRE containing genes in response to those stresses (Nakashima et al., 2000). However, when DREB2 was constitutively overexpressed in Arabidopsis plants, only weak induction of the CRT/DRE-containing target genes was observed, and the plants did not show a significant increase in the level of stress tolerance (Liu et al., 1998). It has been speculated that the activity of the DREB2 proteins is regulated at the post-transcriptional level (Shinozaki and Yamaguchi-Shinozaki, 2000). Here, we show that the overexpression of CBF4 alone is sufficient to provide increased dehydration protection,

and we propose that the CBF4 gene also plays a role in the signal transduction of drought adaptation in Arabidopsis plants (Fig. 6). A recent study by Sakuma et al. (2002) showed that *CBF4* (*DREB1d*) expression is induced by salt, but not by drought, cold or abscisic acid under their experimental conditions. It remains to be investigated whether the different experimental procedures have resulted in these discrepancies.

In Arabidopsis, many genes are induced in response to low temperature and water deficit (Pearce, 1999, Shinozaki and Yamaguchi-Shinozaki, 2000, Xiong and Zhu, 2001). Previous studies have shown that multiple transcription factors and *cis*-acting regulatory elements are involved in controlling expression of these genes (Fig. 6). It has been proposed that the ABA independent cold and drought responsive gene expression is regulated by CBF/DREB1 and DREB2 proteins, respectively (Fig. 6) (Medina et al., 1999; Gilmour et al., 1998; Shinwari et al., 1998, Nakashima et al., 2000). On the other hand, the ABA dependent dehydration response involves the ABRE and MYC/MYB promoter elements recognized by bZIP and MYC/MYB transcription factors, respectively (Uno et al., 2000, Razik and Quatrano, 1997, Kim et al., 2001, Abe et al., 1997). Here, we show for the first time that the ABA dependent pathway can also involve the CRT/DRE elements and AP2 type transcription factors (Fig. 6). This observation is in agreement with an earlier study which suggests that the CRT/DRE elements are involved in ABA signal transduction because the ABRE element in the promoter of COR78a/RD29a is not sufficient by itself to elicit an ABA response, and that proper ABA response requires the presence of a region containing the CRT/DRE elements (Yamaguchi-Shinozaki and Shinozaki, 1994). It still remains unclear why the CRT/DRE

element by itself does not appear to be sufficient for ABA-dependent gene activation (Yamaguchi-Shinozaki and Shinozaki, 1994).

An analysis of regulatory elements in the 2kb upstream of the *CBF4* gene by PLACE database (Higo et al.,1999; http://www.dna.affrc.go.jp/htdocs/PLACE/) uncovered several putative bZIP and MYB/MYC binding sites. However, because most of the cis-acting elements known to be involved in bZIP or MYB/MYC binding are degenerate 5 to 8-mers, and occur in several thousand Arabidopsis promoters, sequence analysis alone is insufficient to draw any conclusions. A more detailed functional analysis of the CBF4 promoter is needed to identify its key regulatory elements.

CBF1, 2 and *3* are present in the genome as a tandem array on chromosome 4 (Gilmour et al, 1998; Medina et al., 1999) while the gene encoding their closest related homologue, CBF4, is present on chromosome 5. Phylogenetic analysis (Fig. 1a) suggests that the ancestor of these four genes underwent a gene duplication event that gave rise to *CBF4* and a homologue that subsequently underwent more recent duplication events that produced *CBF1*, *2* and *3*. The fact that the *CBF4* promoter region shows little sequence identity with the promoter regions of *CBF 1*, *2* and *3* (data not shown), which show considerable sequence identity among themselves (Shinwari et al., 1998), suggests that duplications at the *CBF1*, *2* and *3* locus occurred after differences in regulation were established (or well underway). It has been suggested that one way through which genetic diversity can be generated is by the duplication of genome segments and the subsequent rearrangements (Bancroft, 2000). Promoter scrambling via transposable elements causing insertions or deletions has been shown to be a particularly effective way of generating diversity in the promoter regions (Robins and Samuelson, 1992, Wessler et al.,

1995, Kidwell and Lisch, 1997, Britten, 1996, Kloeckener-Gruissem and Freeling, 1995). Many traits of agronomic importance have evolved through changes in the expression of regulatory genes (Doebley, 1998, Lukens and Doebley, 2001), and in many cases, those changes were due to mutations in the promoters rather than the coding regions (Wang et al., 1999, Zhang et al., 2000, Doebley, 1998, Frary et al., 2000). Because the difference between CBF4 and CBF1, 2, and 3 does not appear to have resulted in the selection of fundamentally new functions at the respective loci, we suggest that gene duplication and then promoter evolution have resulted in different CBF loci with conserved protein function but divergent regulatory elements that are responsive to pertinent environmental cues.

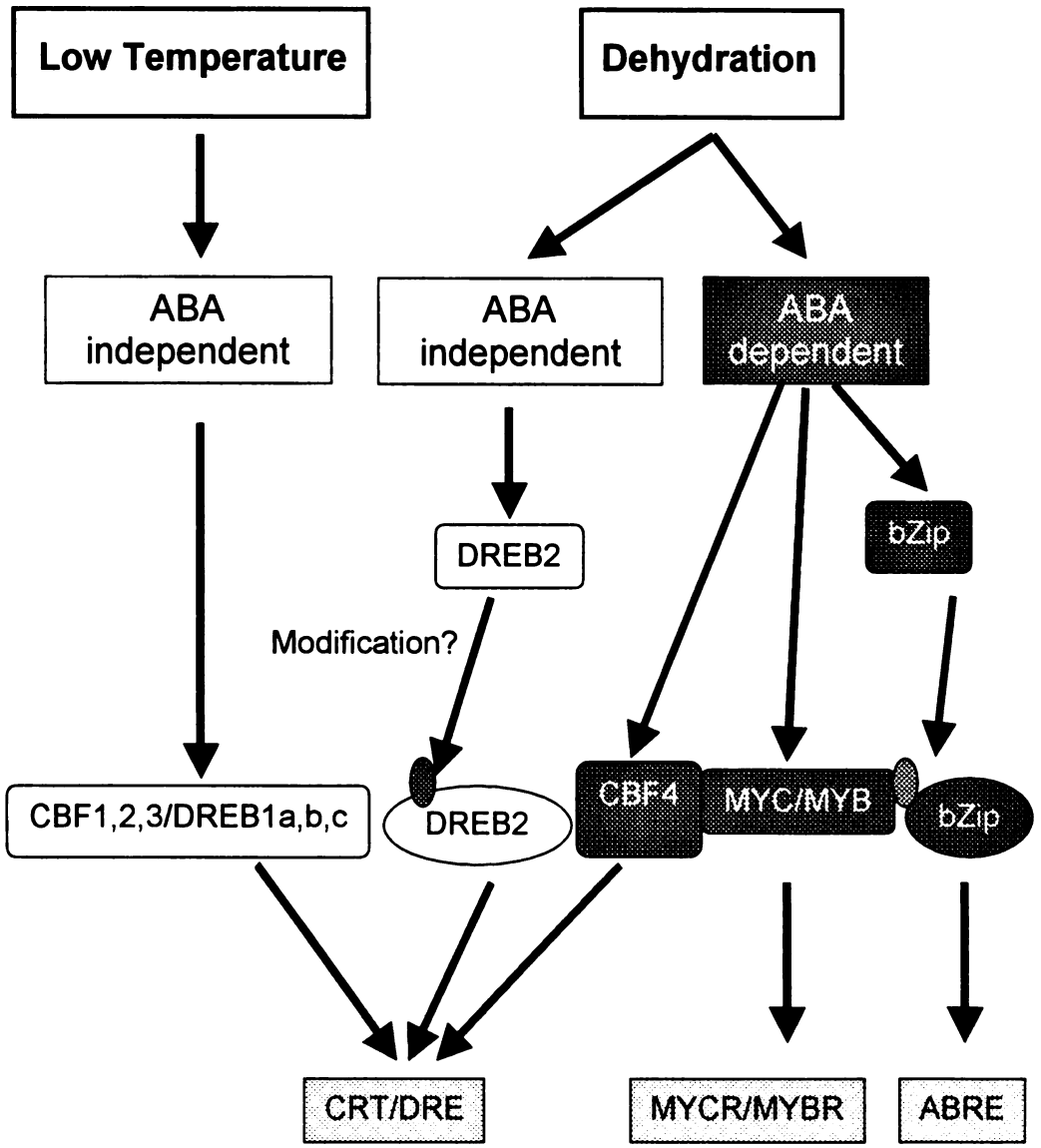

Figure 2.6

Simplified model for the regulation of cold and drought responsive gene expression

Material and Methods

Plant growth

For all experiments requiring plate grown plant material, seeds were vapor sterilized with chlorine gas for 90 minutes, then suspended in a 0.15% (w/v) agarose solution and stratified for 2-4 days at 4°C. For selection of transformed plants, seeds were sown to 80% (w/v) Murashige and Skoog supplemented with 0.3% (w/v) sucrose and 50mg l^{-1} kanamycin. Kanamycin resistant plants were transplanted to soil after 7 days. All plants were grown under continuous light with a light intensity of approximately 100μE at 22°C in either environmental control chambers or growth rooms. Cold treatment of plants was done at 4°C under constant illumination from cool white fluorescent lights (approximately 35 mmole $m^{-2}sec^{-1}$).

Constructs and plant transformation

The 5' and 3' ends of CBF4 was determined by RACE (Rapid Amplification of cDNA Ends) following the manufacture's instructions (Marathon and SMART RACE systems, CLONTECH). The CBF4 gene was amplified by PCR from cDNA using primers 5'-GCACGCGTCGACCATCTTATCCAAAGAAAAAATGAATCC and 5' GGGAAAGCGGCCGCAACTTATTATCCAGAAAAAGAGCCAAAAAA. The cDNA was derived from a mixed mRNA population from several tissues including shoot, root, flower, rosette leaf, cauline leaf, silique, germinating seed and a variety of conditions including treatment with auxin (1μM 2,4-D), abscisic acid (50μM), cold (4°C), mannitol (3M), heat (37°C), sodium chloride (200mM), pathogen infection (Erysiphe orontii and Fusarium oxysporum), salicylic acid (0.5mM), and drought. The 720 bp product

containing the entire coding region of CBF4 was cloned as SalI-NotI fragment downstream of the 35S CaMV promoter in a standard binary transformation vector containing a kanamycin resistance selectable marker. The plasmid was introduced into *Agrobacterium tumefaciens* by electroporation. Arabidopsis plants (Columbia accession) were transformed by the floral dip method (Clough and Bent, 1998). Agrobacteria infiltration of tobacco leaves were carried out essentially the same as described in Yang et al (2000), except that the CBF genes and the COR78 promoter::GUS constructs were cloned in separate plasmids.

Plant material and treatments

Arabidopsis thaliana ecotype columbia was grown in a percival series 101 (Percival Scientific, Inc., Perry, Iowa) controlled environment chamber at 22°C under continuous illumination of approximately 100 mmole $m^{-2}sec^{-1}$ for 12-14 days. Plants were grown under sterile conditions in Petri plates according to Gilmour et al., 1998 with the exception that the seeds were germinated on filter paper for drought, ABA and control experiments. Cold treatment was performed by transferring plates to a cold room set at 4°C under constant illumination from cool white fluorescent lights (approximately 35 mmole $m^{-2}sec^{-1}$). For the drought treatment, the filter papers were removed from the plate and placed to dry over desiccant. The ABA treatment was done by removing the filter papers from the plate and placing them in Gamborgs B5 liquid plus100 mm ABA. For controls, the plants on filter papers were transferred to Gamborgs B5 liquid. Tissue was harvested at the indicated times and was immediately frozen in liquid nitrogen before total RNA was extracted.

RNA hybridization and cDNA probes

Total RNA was extracted from Arabidopsis plants as described by Whitelam et al. (1993) with the exception that the last lithium chloride extraction was omitted. Northern transfers were prepared and hybridized as described by Hajela et al. (1990) and washed at high stringency with the temperature being 60°C instead of 50°C (Stockinger et al., 1997). Full length cDNA's of CBF4, CBF1, COR15a, and eIF4a were labeled with P-32 with the random primers DNA labeling system (Gibco BRL) as directed by the manufacturer.

Stress tolerance assays

In the whole plant freezing assay, 2 week old plants grown on petri dishes, with or without cold acclimation for 4 days at 4°C, were frozen for 20 hours at -15°C (cold-acclimated plants) or -10°C (non-acclimated plants), respectively. Plant survival was scored after two days of recovery under normal growth conditions. Electrolyte leakage assays were performed essentially as described (Gilmour et al., 2001) with a minor modification: Instead of leaves, 2-4 complete 2 week old plants grown on Petri dishes were used per tube. For the drought stress evaluation, pots (10 cm diameter) were filled with a 1:1 (v/v) vermiculite:perlite mix. To ensure even plant growth a thin (1cm) layer of promix soil (Hummert International, Earth City, Missouri) was added. Seedlings were grown for 2 weeks with constant watering before the water was withheld. After 9 days without water all the pots were re-watered simultaneously and the plant re-growth was scored four days later.

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Chapter 3

A Major Role for the CBF Cold Response Pathway in Configuring the Low Temperature Metabolome

SUMMARY

The Arabidopsis CBF cold response pathway has a central role in cold acclimation, the process whereby plants increase their freezing tolerance in response to low nonfreezing temperatures. Here we examined the changes that occur in the Arabidopsis metabolome in response to low temperature and the role of the CBF cold response pathway in bringing about these changes. The results indicate that extensive changes occur in the metabolome of plants exposed to low temperature and that these changes can be largely mimicked by over-expression of the CBF3 transcriptional activator. However, a dichotomy was evident regarding the effects that CBF over-expression had on the levels of polar and non-polar metabolites. Of the 326 polar metabolites that were identified as increasing in response to cold acclimation in Arabidopsis ecotype Ws, 258 (79%) were found to increase in response to CBF3 over-expression. In contrast, only 1 (5%) of the 21 non-polar metabolites that were found to increase in cold-treated plants increased in response to CBF3 over-expression. Additional experiments showed that Cvi ecotype of Arabidopsis was less freezing tolerant than the Ws ecotype and that the low temperature metabolome of Cvi was depleted in metabolites affected by CBF over-expression. Significantly, cold-induced expression of the *CBF* genes was much lower in ecotype Cvi as compared to ecotype Ws.

Taken together, these results indicate a major role for the CBF cold response pathway in configuring the low temperature metabolome of Arabidopsis.

INTRODUCTION

Many plants have the ability to sense low temperature and respond by activating mechanisms that lead to an increase in freezing tolerance. This adaptive response is known as cold acclimation. At present, the best understood genetic system that has a role in cold acclimation is the Arabidopsis CBF cold response pathway. Exposing Arabidopsis plants to low temperature results in rapid induction of a small family of transcriptional activators known either as *CBF1*, *2* and *3* (Gilmour et al., 1998; Medina et al., 1999) or *DREB1b*, *c* and *a*, respectively (Liu et al., 1998). These transcription factors, which belong to the AP2 domain family of DNA binding proteins, recognize a *cis*-acting regulatory element known as the CRT (C-repeat)/DRE (dehydration response element) (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994) that is present in the promoters of many cold-inducible genes such as *COR15a* and *COR78* (also known as *RD29A* and *LTI78*) (Stockinger et al., 1997). Expression of the more than 30 genes that comprise the CBF regulon, i.e., those that are induced in response to CBF expression (Fowler and Thomashow, 2002), then results in an increase in freezing tolerance.

This increase in freezing tolerance involves multiple mechanisms including the production of cryoprotective polypeptides, such as COR15a (Jaglo-Ottosen et al., 1998), and synthesis of compatible solutes that have cryoprotective properties including proline, sucrose and raffinose (Gilmour et al., 2000). Synthesis of these metabolites is brought about by CBF regulon genes that encode enzymes that catalyze rate limiting steps for the

synthesis of these molecules. In the case of raffinose and proline, this includes induction of genes encoding galactinol synthase and pyrroline-5-carboxylate synthase, respectively (Gilmour et al., 2000; Taji et al., 2002).

To what extent is the cold acclimation program in Arabidopsis driven by the CBF transcription factors? Mutational analyses suggest that the CBF cold response pathway is not the sole genetic system with a role in freezing tolerance. The *eskimo* mutant of Arabidopsis described by Xin and Browse (1998) is constitutively more freezing tolerant than wild-type plants, but the COR genes are not expressed indicating that the mutation activated a pathway outside the CBF system. Similarly, *ada2* mutants of Arabidopsis—ADA2 encodes a transcriptional adaptor protein (Stockinger et al., 2001)—are constitutively more freezing tolerant than wild-type plants, but again, *COR* genes are not constitutively induced (Vlachonasios et al., 2003). Thus, in wild-type plants grown at warm temperature (nonacclimated plants), the ADA2 protein may normally inhibit expression of a freezing tolerance pathway that is distinct from the CBF cold response pathway. Molecular genetic analyses are consistent with the notion that there are freezing tolerance pathways in addition to the CBF cold response pathway. Using Affymetrix Gene Chips representing about 8,000 genes, Fowler and Thomashow (2002) identified transcripts for 218 genes that increased in response to low temperature. Of these, at least 60 genes appeared to fall outside the CBF regulon as transcripts for these genes were unaffected by overexpression of either CBF1, 2 or 3 in transgenic Arabidopsis plants. Additional microarray experiments are consistent with these findings.

Recent genetic evidence further supports the role of CBF in the process of cold acclimation. Natural variation for freezing tolerance in Arabidopsis thaliana ecotypes, Ler and Cvi, was used to identify quantitative trait loci important to this trait. This work led to the identification of a QTL on chromosome 4 that maps to the CBF genes (Martinez-Zapater et al., 2002).

Global approaches to analyze biological systems have become a central theme in current research applications. Gene profiling via microarray or Affymetrix Gene Chips have been used to analyze the transcriptome of biological systems (Richmond and Somerville, 2000). This provides a first step toward understanding how an organism may respond to a stimulus. Anlaysis of the transcriptome is only one of many global approaches being developed. Metabolomics is another global approach to identify all the metabolites, the metabolome, of an organism (Fiehn, 2002). Gas chromatography (GCMS) and reverse phase liquid chromatography (LCMS) both coupled with mass spectrometry are two tools that can be used to help identify the metabolome of an organism (Fiehn et al., 2000; Roessner et al., 2000). GCMS with the extraction method used detects metabolites that are more polar while reverse phase LCMS with the extraction method used detects metabolites that more non-polar. Recently, 326 distinct metabolites in Arabidopsis were identified by GCMS. In this study the metabolome of two different Arabidopsis ecotypes, Col and C-24, were compared with two different mutants, *dgd1-1*, and *sdd1-1* (Fiehn et al., 2000). This technology was also recently used to characterize transgenic potato tubers altered in carbon metabolism (Roessner et al., 2001). LCMS has also been used in a wide variety of applications to identify and characterize metabolites (Fiehn, 2002).

Progress in understanding the biochemical/metabolic changes associated with cold acclimation has lagged behind the characterization of the molecular/genetic changes associated with cold acclimation. A fundamental question in regard to understanding the process of cold acclimation is the identification of all the metabolic changes associated with cold acclimation. Furthermore it is important to determine the contribution of CBF to these metabolic changes. In addition, it would be insightful to compare the metabolic changes influenced by cold acclimation in Arabidopsis ecotypes that exhibit natural variation in freezing tolerance.

Here we provide a thorough example of how metabolomics can be applied to the complex biological phenomenon of cold acclimation and the role of the CBF cold response pathway. In this study, we examine the effects that low temperature and CBF over-expression have on the metabolome of Arabidopsis. The results indicate that extensive changes in metabolite levels occur in response to low temperature and that these changes can be largely mimicked by CBF over-expression. In addition, Arabidopsis Cvi plants were found to express *CBF* genes at a low level in comparison to Ws ecotype plants. In addition, the Cvi plants were less freezing tolerant than Ws plants and had a low temperature metabolome that was depleted of metabolites affected by CBF overexpression. Taken together, the results indicate a prominent role for the CBF cold response pathway in configuring the low temperature metabolome of Arabidopsis.

RESULTS

Cold Acclimation Involves Extensive Changes in the Arabidopsis Metabolome

Metabolic changes associated with cold acclimation in Arabidopsis ecotype Ws were determined by comparing the metabolomes of non-acclimated and cold-acclimated plants. Metabolite levels were determined by GCMS and reverse phase LCMS. In the GCMS analysis, a total of 438 low molecular weight carbohydrates, amines, organic acids and other polar molecules were identified. Of these, a large number, 326 (74%), were found to increase in cold-acclimated plants ($p < 0.001$) (Table 3.1). The increases, measured as changes in peak area, varied from less than 2-fold to greater than 25-fold, with 113 (35%) of the metabolites increasing 5-fold or more (Table 3.1). Among these metabolites that increased at least 5-fold, several had previously been shown to increase in response to cold acclimation in either Arabidopsis or other plants. These included glucose, fructose, raffinose, sucrose, trehalose, putresceine, and proline (Gilmour et al., 2000) (Table 3.2). Among these, a number have been shown to have cryoprotective activities including raffinose and proline (Strauss and Hauser, 1986; Anchordoguy et al., 1987). Significantly, 83 of the metabolites that increased more than 5-fold with cold acclimation were unidentifiable (Table 3.2). These are metabolites with possible important roles in freezing tolerance.

The results of the reverse phase LCMS analysis also indicated that significant changes occurred in the non-polar metabolome of Arabidopsis ecotype Ws with cold acclimation (Table 3.3). Of the 61 flavonoid, lipid, and other non-polar molecules tested, 21 (34%) were found to increase in levels ranging from less than 2 to greater than 25 fold

Table 3.1.

Numbers of metabolites in Arabidopsis detected by GCMS that increase in response to low temperature .

Peak Area Change in Cold in Ecotype Ws	Carbohydrates (118)	Amines (77)	Acids (47)	Other Compounds (196)	All Metabolites (438)
>25	14	3	0	9	**26**
>5 to 25	15	28	9	35	**87**
>1 to 5	62	28	23	100	**213**
Total	**91**	**59**	**32**	**144**	**326**

Metabolites were selected as increasing in response to low temperature if they met the following criteria. First, a measured increase in peak area of a metabolite, calculated by dividing the average peak area of the cold acclimated samples by the average peak area of the non-acclimated samples. Second, a significant difference in peak area between cold acclimated samples and non-acclimated samples for each metabolite as measured by a p-value of <0.001 as calculated by a t-test.

Table 3.2.

A list of metabolites from Table 3.1 that increase in response to cold acclimation in Arabidopsis with a peak area change greater than 5.

Carbohydrates	Amines		Acids	Other Compounds
Fructose[1,2]	Asparagine[1]	Ornithine[1]	Ascorbate[1]	44 Unidentifieds (42[1]/9[2]/8[1,2])
Glucose[1,2]	Aspartate[1]	Citrulline[1]	Aconitate	
Inositol[1]	Glycine[1]	Putrescine[1]	Alpha-ketoglutarate	
Melibiose[1]	Glutamine[1,2]	Threitol[1]	Isosuccinate[1]	
Sucrose[1]	Glutamate[1]	Tryptophan	Itaconate[1]	
Trehalose	Proline[1,2]		Malate[1]	
Galactinol[1,2]	Serine[1]		Gluconate[1]	
Raffinose[1,2]	Cycloserine		Gluconate lactone[1]	
18 Unidentified Carbohydrates (15[1]/9[2]/7[1,2])	Homoserine[1]		Unidentifed Acid[1]	
3 Unidentified Oligo-Carbohydrates (3[1]/1[2])	17 Unidentified Amines (14[1]/1[2]/1[1,2])			

[1] indicates that the metabolite is influenced by CBF3 over-expression
[2] indicates that the metabolite has a peak area change >25

Table 3.3.

Numbers of metabolites detected from reverse phase LCMS that increase in response to low temperature

Peak Area Change in Cold in Ecotype Ws	Flavanoids (7)	Lipids (26)	Other Compounds (29)	Total (63)
>25	0	0	1	**1**
>5 to 25	2	0	2	**4**
>1 to 5	3	6	7	**16**
Total	**5**	**6**	**10**	**21**

Metabolites were selected as increasing or decreasing in response to low temperature if they met the following criteria. First, a measured increase in peak area of a metabolite calculated by dividing the average peak area of the cold acclimated samples by the average peak area in the non-acclimated samples. Second, a significant difference in peak area between cold acclimated samples and non-acclimated samples for each metabolite as measured by a p-value of <0.05 as calculated by a t-test.

($p < 0.05$). Of these, 13 (62%) were unidentified and thus again, potentially represent novel molecules with important roles in acclimation to low temperature (Table 3.4).

The Metabolome of Non-acclimated Transgenic Plants that Constitutively Express the CBF Regulon Closely Mimics the Low Temperature Metabolome of Wild-Type Plants

As alluded to previously, the CBF regulon includes genes involved in the synthesis of compatible solutes with cryoprotective activities such as raffinose, sucrose, and proline (Gilmour et al., 2000). A fundamental question thus raised is to what extent is the low temperature metabolome of Arabidopsis configured by activation of the CBF regulon of genes? To address this question, we compared the metabolomes of nonacclimated transgenic Arabidopsis plants that over-express CBF3 (transgenic lines A28 and A30) with non-acclimated and cold-acclimated wild-type Arabidopsis Ws plants. Again, both GCMS and LCMS were used for the analysis.

Of the 326 polar metabolites that were identified as increasing in response to cold acclimation, 258 (79%) were also found to increase in response to CBF3 over-expression in nonacclimated transgenic plants (Table 3.5). Moreover, of the 113 metabolites that increased more than 5-fold in response to low temperature, 101 (89%) were found to increase in response to CBF over-expression (Table 3.2). These results suggested that the metabolome of CBF3 over-expressing plants closely resembled that of cold-acclimated plants. To test this notion, the levels of the 438 polar metabolites that were determined in nonacclimated CBF3 over-expressing transgenic plants, nonacclimated wild-type plants and cold-acclimated wild-type plants and the results were subjected to hierarchical cluster

Table 3.4.

A list of Metabolites from Table 3.3 that increase in response to cold acclimation in Arabidopsis

Flavanoids	**Lipids**	**Other Compounds**
Hesperidin	1 DGDG	Glucosinolate
4 Kaempferols	Sulfolipid[1]	9 Unidentifieds (2^2)
	4 Unidentified Lipids	

[1] indicates that the metabolite is influenced by CBF3 over-expression
[2] indicates that the metabolite has a peak area change >25

Table 3.5.

Numbers of metabolites in Arabidopsis detected by GCMS that increase in response to low temperature and the number of those metabolites that increase in transgenic plants over-expressing CBF3

Peak Area Change in Cold Ecotype Ws (p-value <0.001)	All Metabolites (438)	
	Total	CBF Influenced
>1	326	258 (79%)

Metabolites were selected as increasing in response to low temperature if they met the following criteria. First, a measured increase in peak area of a metabolite calculated by dividing the average peak area of the cold acclimated samples by the average peak area of the non-acclimated samples. Second, a significant difference in peak area between cold acclimated samples and non-acclimated samples for each metabolite as measured by a p-value of <0.001 as calculated by a t-test.

Of the 326 cold induced metabolites, they were selected as being influenced by CBF over-expression if they met the following criteria in two independent transgenic lines. First, an increase in peak area of a metabolite calculated by dividing the average peak area of CBF3 transgenic lines by the average peak area of non-acclimated samples. Second, a significant difference in peak area between CBF3 transgenic lines and non-acclimated samples for each metabolite as measured by a p-value of <0.001 as calculated by a t-test

analysis. The analysis revealed that the polar metabolome of the non-acclimated CBF3 expressing transgenic plants more closely resembled that of cold-acclimated wild-type plants than that of non-acclimated wild-type plants (Figure 3.1).

Significantly, the opposite picture emerged regarding the role of the CBF regulon in determining the composition of the low temperature non-polar metabolome. Of the 22 non-polar metabolites that were found to increase in response to low temperature, only 1, a sulfolipid, was found to increase in response to CBF over-expression (Table 3.6). Thus, as would be expected, hierarchical cluster analysis of the levels of the 61 non-polar metabolites in the various plants indicated that the non-polar metabolome of the CBF3 over-expressing plants was more similar to that of non-acclimated wild-type plants than it was to that of cold-acclimated wild-type plants (Figure 3.2). These results indicated that the CBF regulon has a minor role in determining the composition of the non-polar metabolome at low temperature.

Arabidopsis ecotype Cvi is Less Freezing Tolerant than Arabidopsis ecotype Ws and Has a "Weak" CBF Locus and Low Temperature Metabolome Devoid of Many Metabolites Controlled by the CBF Regulon

Arabidopsis ecotype Cvi, which was collected from the Cape Verde islands, is less freezing tolerant than the Ler ecotype, which was collected in Germany. To understand the genetic basis of this natural variation, Salinas and colleagues conducted a QTL analysis of freezing tolerance in recombinant inbreed lines derived from a cross between these two ecotypes. The results indicated that a major QTL for freezing tolerance mapped to the CBF locus (Martinez-Zapater et al., 2002) thus raising the

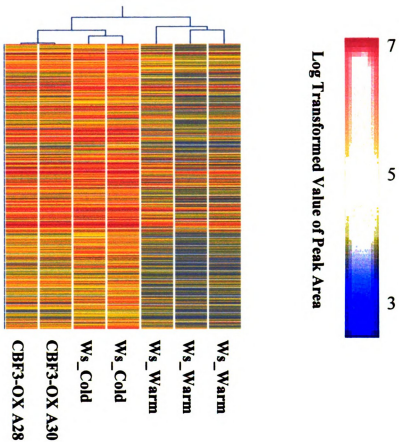

Figure 3.1.

Hierarchical Clustering of the polar (GCMS) metabolomes from non-acclimated, cold-acclimated, and transgenic plants over-expressing CBF3

The mean log transformed values of the peak area of each metabolite from the individual samples was calculated, and the metabolomes from each treatment were clustered using a Pearson correlation. The metabolites are listed alphabetically.

Table 3.6.

Numbers of metabolites in Arabidopsis detected by LCMS that increase in response to low temperature and the number of those metabolites that increase in transgenic plants over-expressing CBF3

Peak Area Change in Cold Ecotype Ws (p-value <0.001)	All Metabolites (62)	
	Total	CBF Influenced
>1	22	1 (4%)

Metabolites were selected as increasing in response to low temperature if they met the following criteria. First, a measured increase in peak area of a metabolite calculated by dividing the average peak area of the cold acclimated samples by the average peak area of the non-acclimated samples. Second, a significant difference in peak area between cold acclimated samples and non-acclimated samples for each metabolite as measured by a p-value of <0.05 as calculated by a t-test.

Metabolites were selected as being influenced by CBF over-expression if they met the following criteria in two independent transgenic lines. First, an increase in peak area of a metabolite calculated by dividing the average peak area of CBF3 transgenic lines by the average peak area of non-acclimated samples. Second, a significant difference in peak area between CBF3 transgenic lines and non-acclimated samples for each metabolite as measured by a p-value of <0.05 as calculated by a t-test

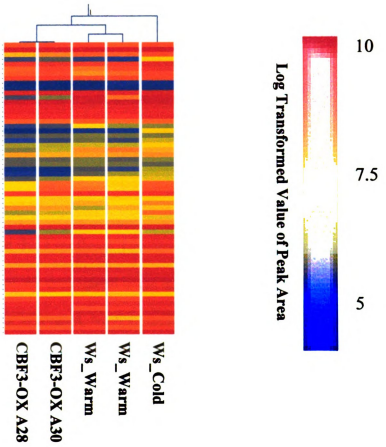

Figure 3.2.

Hierarchical Clustering of the non-polar (reverse phase LCMS) metabolomes from non-acclimated, cold-acclimated, and transgenic plants over-expressing CBF3

The mean log transformed values of the peak area of each metabolite from the individual samples was calculated, and the metabolomes from each treatment were clustered using a Pearson correlation. The metabolites are listed alphabetically.

possibility that the difference in freezing tolerance between Cvi and Ler was due to the CBF locus of Cvi being "weaker" than that of Ler.

The results of Salinas and colleagues (2002) led us to speculate that natural variation at the CBF locus might also lead to differences in low temperature metabolome conditioned by the CBF locus. That is, if the CBF locus of Cvi was weak in comparison to that of Ler, then changes in the metabolome that fell under control of the CBF regulon might not be as highly expressed, which in turn, might result in a decrease in freezing tolerance. To test this hypothesis, we chose to compare the low temperature metabolome of Cvi with that of Ws determined in the experiments described above. However, to place these results in context, we first needed to determine whether Cvi was less freezing tolerant than Ws and to gain some insight into whether the CBF locus of Cvi might be weaker than that of Ws. To address the first issue, whole plant freezing tolerance experiments were conducted. The results indicated that Cvi was indeed less freezing tolerant than Ws; when Ws plants were cold-acclimated for 14 d and then subjected to a freeze to -5°C they survived, whereas Cvi plants treated in an equivalent manner did not (Figure 3.3).

To address the issue of CBF locus expression, transcript levels for the CBF genes and downstream CBF regulon genes were determined in Cvi and Ws plants that were exposed to low temperature. The results were consistent with the notion that the CBF locus of Cvi was weaker than that of Ws. In particular, CBF transcripts accumulated to much higher levels in Ws than they did in Cvi (Figure 3.4). This was also true of the CBF-targeted genes, *COR15a*, *GolS*, and *P5CS*. *GolS* encodes galactinol synthase, a rate limiting step in the synthesis of raffinose (which increases dramatically in response to

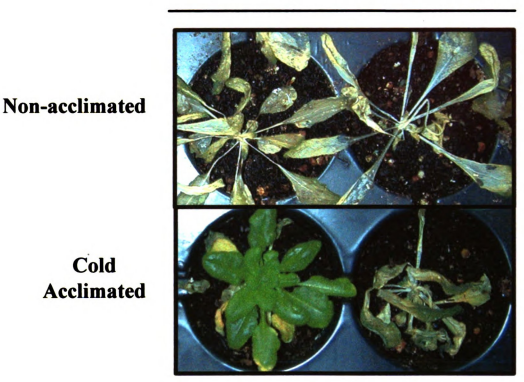

Figure 3.3.

Whole plant freeze test showing freezing tolerance of cold acclimated Ws and sensitivity of cold acclimated Cvi.

Arabidopsis thaliana ecotypes Ws and Cvi were frozen at -2°C for 1 day then -5°C for 3 days and then allowed to recover for 4 days. Non-acclimated plants were grown for 45 days and acclimated plants were grown for 41 days followed by 14 days of cold acclimation at 4°C.

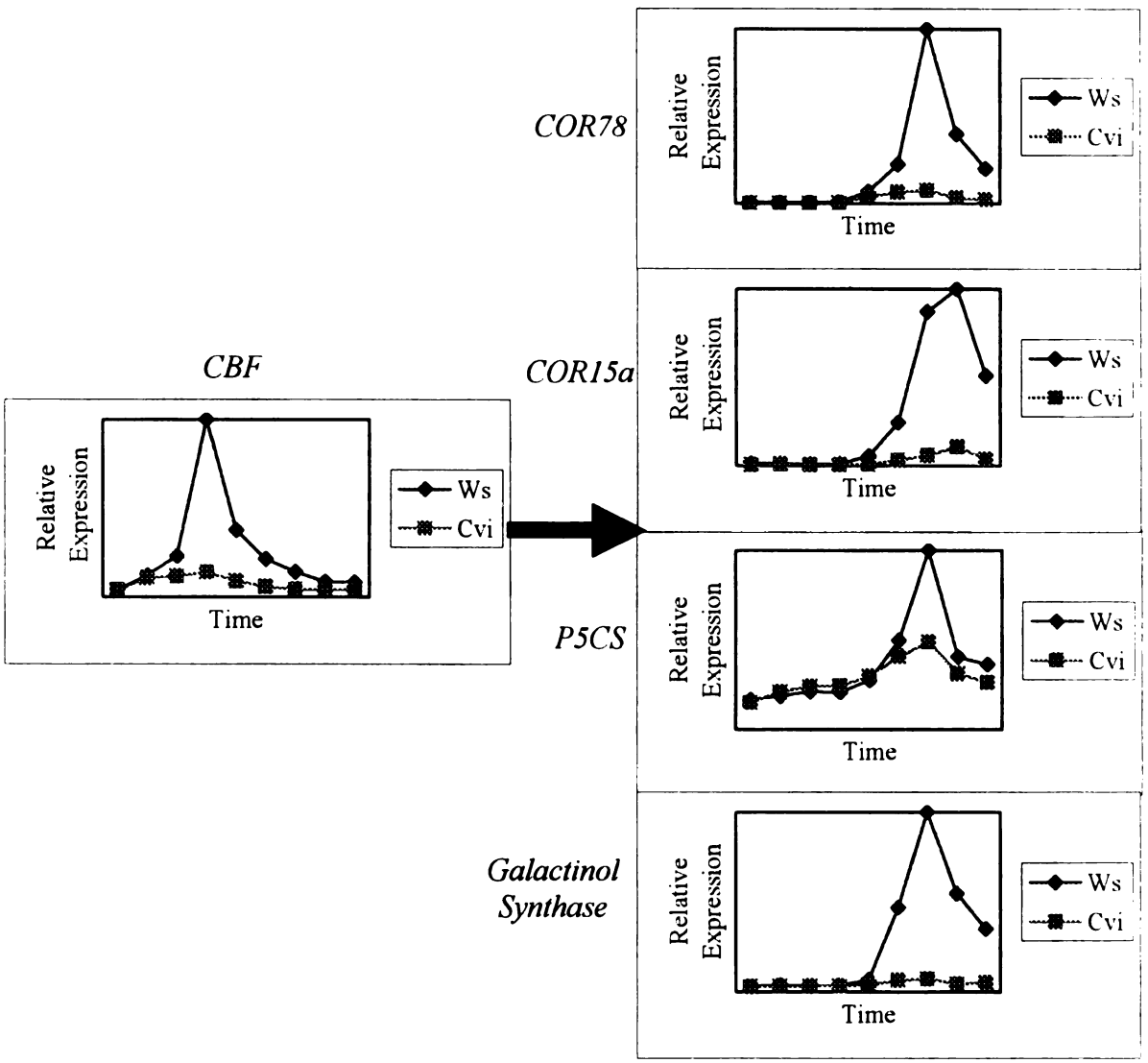

Figure 3.4

Relative gene expression levels of *CBF*, *COR15a*, *COR78*, *pyrroline 5-carboxylate synthase, and galactinol sythase* in response to low temperature for the indicated times in Arabidopsis thaliana ecotypes Ws and Cvi.

Northern Blots were performed for each of the indicated genes. Expression levels were normalized to ribosomal RNA to account for differences in loading and then plotted as a percentage of the highest point of induction (100%) on the y-axis to represent the relative expression of each gene.

Time points from left to right on the x-axis are 0 h, .5h, 1h, 2h, 4h, 8h, 24h, 3d, and 7d (h=hours, d=days)

low temperature; e.g., see Table 3.2) (Figure 3.4) while *P5CS* encodes pyrroline 5-carboxylate synthase, the rate limiting step in the synthesis of proline (which increases dramatically in response to low temperature; e.g., see Table 3.2) (Figure 3.4). In contrast, there was little difference in the cold-induced transcript levels of *PAL* (phenylalanine ammonia lyase) and *CS* (chalcone synthase), two genes that are not affected by CBF expression (Figure 3.5).

To test the hypothesis that a weak CBF locus in Cvi may influence its low temperature metabolome when compared to Ws, we first took a targeted analysis of the metabolites proline, galactinol, and raffinose. We reasoned that the CBF locus in Cvi that was reflected in decreased transcript accumulation of the CBF target genes galactinol synthase and pyrroline 5-carboxylate synthase may result in differences in the accumulation of the metabolites synthesized by these enzymes. To test this we investigated the accumulation of galactinol, raffinose, and proline in ecotypes Ws and Cvi in response to cold acclimation. These metabolites all increased in response to low temperature in Cvi; however their level of accumulation was different than Ws. We found that the levels of galactinol and raffinose in Cvi were three to five percent of the levels in Ws (Figure 3.6). Furthermore we found the levels of proline in Cvi were 50 percent of the levels in Ws (Figure 3.7). Interestingly, the percent accumulation of these metabolites in response to low temperature in Cvi is very similar to the percent transcript accumulation (Figure 3.4). We have established that the CBF locus in Cvi affects the accumulation of three metabolites where the enzymes responsible for their biosynthesis are regulated by CBF.

Sucrose Synthase

Phenyalanine Ammonia Lyase

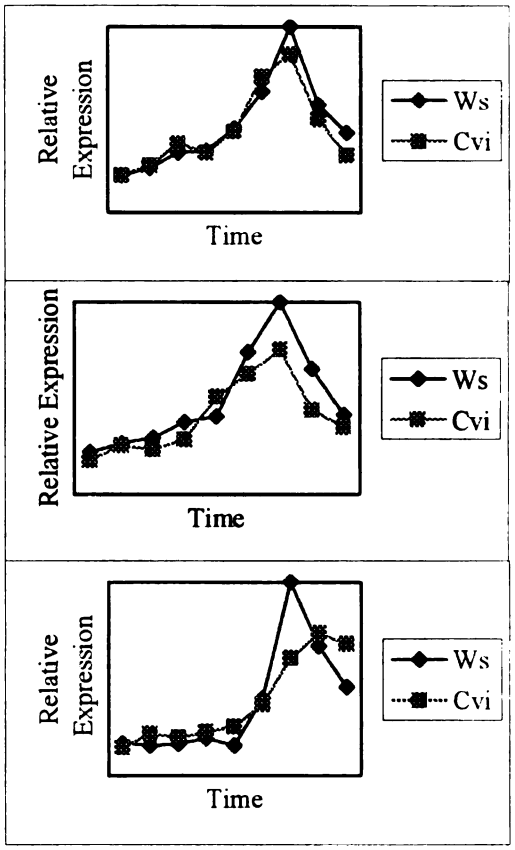

Figure 3.5.

Relative gene expression levels of *sucrose synthase*, *phenylalanine ammonia lyase*, and *chalcone synthase* in response to low temperature for the indicated times in Arabidopsis thaliana ecotypes Ws and Cvi.

Northern Blots were performed for each of the indicated genes. Expression levels were normalized to ribosomal RNA to account for differences in loading and then plotted as a percentage of the highest point of induction (100%) on the y-axis representing the relative expression of each gene.

Time points from left to right on the x-axis are 0 h, .5h, 1h, 2h, 4h, 8h, 24h, 3d, and 7d (h=hours, d=days)

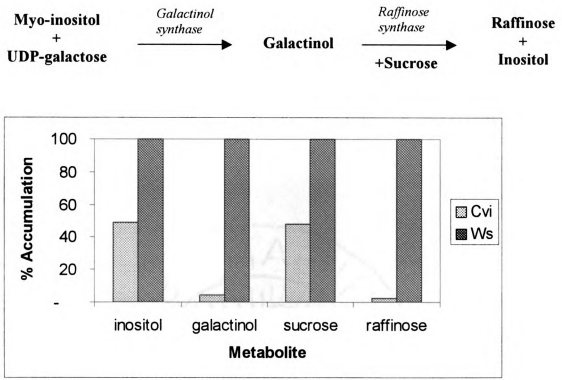

Figure 3.6.

The accumulation of metabolites in the raffinose biosynthetic pathway comparing Arabidopsis ecotypes Ws and Cvi.

Metabolites were defined as increasing to a greater level in Ws than Cvi if they met the following criteria. First, the metabolite was identified as being cold regulated in ecotype Ws. Second, it increased to a greater level in ecotype Ws than Cvi calculated by dividing the average peak area of each metabolite in cold acclimated Ws by the average peak area of that same metabolite in ecotype Cvi. Third, the difference in peak area between the cold acclimated samples in each ecotype was significant as measured by a p-value of <0.001 calculated by a t-test.

The percent accumulation of a metabolite in ecotype Cvi was calculated by dividing the average peak area from the Cvi cold acclimated sample by the average peak area from the Ws cold acclimated sampled and then multiplied by 100.

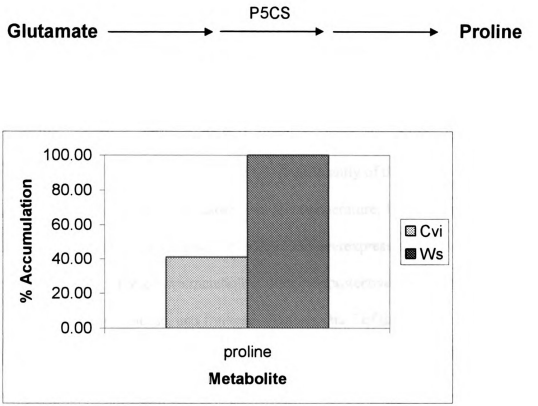

Figure 3.7.

The accumulation of proline comparing Arabidopsis ecotypes Ws and Cvi.

Metabolites were defined as increasing to a greater level in Ws than Cvi if they met the following criteria. First, the metabolite was identified as being cold regulated in ecotype Ws. Second, it increased to a greater level in ecotype Ws than Cvi calculated by dividing the average peak area of each metabolite in cold acclimated Ws by the average peak area of that same metabolite in ecotype Cvi. Third, the difference in peak area between the cold acclimated samples in each ecotype was significant as measured by a p-value of <0.001 calculated by a t-test.

The percent accumulation of a metabolite in ecotype Cvi was calculated by dividing the average peak area from the Cvi cold acclimated sample by the average peak area from the Ws cold acclimated sampled and then multiplied by 100.

To further test this hypotheses we identified metabolites like proline, galactinol, and raffinose that change in response to low temperature in Ws but accumulate to greater levels in Ws when compared to Cvi. We speculated that a weak CBF locus in Cvi may result in decreased levels of many of the 258 metabolites that were influenced by CBF and increased in response to low temperature in Ws (Table 3.5). Of the 326 metabolites that change in response to low temperature in ecotype Ws (Table 3.1), 95 accumulate to a greater level in Ws ($p < 0.001$), 79 (83%) of them were influenced by CBF over-expression ($p < 0.001$) (Table 3.7 and 3.8). Significantly of the 26 metabolites that have a fold change greater than 25 in response to low temperature, 19 accumulate to a greater level in Ws, 18 of them being mimicked by CBF overexpression (Table 3.7). Among these are many of the known metabolites with cyroprotective properties including proline, raffinose, glucose, and fructose. Furthermore 7 of the 18 metabolites accumulate to 10 percent of the levels in Ws, including galactinol and raffinose (Figure 3.8). In addition another 11 of the 18 metabolites, including proline accumulate to 50 percent of the levels in Ws (Figure 3.8). The quantitative difference in many of the metabolites between Ws and Cvi provides further support to the role of CBF in configuring the low temperature metabolome. Furthermore, these differences in accumulation may explain some of the difference in freezing tolerance between the two ecotypes.

Table 3.7.

Metabolites that are cold regulated in Ws (Table 1) and accumulate to a greater level in Ws than Cvi; also indicating the numbers that are influenced by CBF over-expression

Fold Change in Ws Cold	Carbohydrates		Amines		Acids		Other Compounds		All Metabolites		
	Total	Ws/Cvi (>1)	Total	Ws/Cvi (>1)	Total	Ws/Cvi (>1)	Total	Ws/Cvi (>1)	Total	Ws/Cvi (>1)	CBF Influenced
>25	14	13	3	2	0	0	9	4	26	19	18
>5 to 25	15	9	28	7	9	3	35	9	87	28	24
>1 to 5	62	20	28	4	23	7	100	17	213	48	37
Total	91	42	59	13	32	10	144	30	326	95	79

Metabolites were defined as increasing to a greater level in Ws than Cvi if they met the following criteria. First, the metabolite was identified as being cold regulated in ecotype Ws. Second, it increased to a greater level in ecotype Ws than Cvi calculated by dividing the average peak area of each metabolite in cold acclimated Ws by the average peak area of that same metabolite in ecotype Cvi. Third, the difference in peak area between the cold acclimated samples in each ecotype was significant as measured by a p-value of <0.001 calculated by a t-test.

Table 3.8.

List of metabolites that accumulate greater level in ecotype Ws than ecotype Cvi from Table 3.7

Carbohydrates	Amines	Acids	Other Compounds
Fructose[1,2]	Glutamic acid[1]	Alpha-ketoglutarate	Indole-3AcCN[2]
Glucose[1,2]	Leucine[1]	Ascorbate[1]	Indole derivative[2]
Galactinol[1,2]	Proline[1]	Citrate[2]	28 Unidentifeds ($24^1/7^2/7^{1,2}$)
Raffinose[1,2]	Threonine[1]	Fumarate[1]	
Melibiose[1,2]	Tryptophan[1]	Galactonate[1]	
Inositol[1]	Valine[1]	Gluconate[1,2]	
Rhamnose[1]	7 Unidentified Amines ($4^1/4^2/2^{1,2}$)	Shikamate[1]	
Psciose[1]		3 Unidentified Acids (3^1)	
Lyxose[1]			
Sucrose[1]			
24 Unidentified Carbohydrates ($19^1/9^2/8^{1,2}$)			
8 Unidentified Oligo carbohydrates ($8^1/5^2$)			

[1] indicates that the metabolite is CBF influenced
[2] indicates that the metabolite accumulates to less than 40% in Cvi when compared to Ws

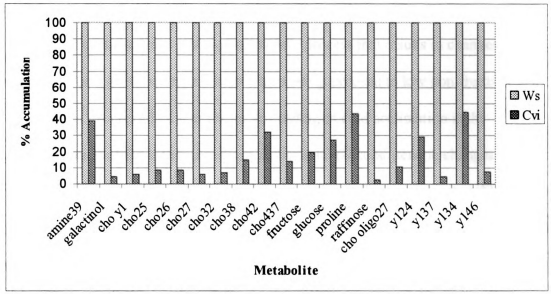

Figure 3.8.

The accumulation of the 19 metabolites that have a fold change greater than 25 and that accumulate to greater levels in Ws comparing Arabidopsis ecotypes Ws and Cvi.

Metabolites were defined as increasing to a greater level in Ws than Cvi if they met the following criteria. First, the metabolite was identified as being cold regulated in ecotype Ws. Second, it increased to a greater level in ecotype Ws than Cvi calculated by dividing the average peak area of each metabolite in cold acclimated Ws by the average peak area of that same metabolite in ecotype Cvi. Third, the difference in peak area between the cold acclimated samples in each ecotype was significant as measured by a p-value of <0.001 calculated by a t-test.

The percent accumulation of a metabolite in ecotype Cvi was calculated by dividing the average peak area from the Cvi cold acclimated sample by the average peak area from the Ws cold acclimated sampled and then multiplied by 100.

Discussion

Cold acclimation is a complex adaptive process that results in changes in metabolism and gene expression from plants being exposed to low non-freezing temperatures. A central goal to the understanding of cold acclimation is the characterization of the biochemical/metabolic and molecular/genetic changes that occur in response to cold acclimation in Arabidopsis thaliana. In recent years, cold acclimation research has principally focused on the changes in gene expression associated with cold acclimation and the signaling events that lead to these changes. This has led to the identification of the CBF/DREB cold response pathway (Fowler and Thomashow, 2002). The central role of the CBF cold response pathway to cold acclimation is supported by the fact that constitutive expression of CBF/DREB results in plants that are constitutively freezing tolerant (Jaglo-Ottosen et al., 1998). Furthermore it is further supported by recent genetic evidence of a QTL that maps to the CBF genes in two Arabidopsis ecotypes that differ in freezing tolerance (Martinez-Zapater et al., 2002). Progress in understanding the biochemical changes that occur in response to cold acclimation and other stimuli has typically lagged behind. Previous work has demonstrated that metabolites such as glucose, fructose, raffinose, and proline increase in response to cold acclimation. It has also been shown that over-expression of the CBF transcription factor results in the subsequent increase in these same metabolites (Gilmour et al., 2000).

In this study, we provide the first global analysis of the metabolic changes associated with cold acclimation. It is of particular importance to note that many of the changes in metabolism in response to cold acclimation may be due to adaptive strategies employed by the plant (Smirnoff, 1998; Bohnert and Sheveleva, 1998) or metabolic

disturbances as a consequence of slower growth and decreased metabolic rates at low temperatures (Bohnert and Sheveleva, 1998). In this study we have only identified the changes that occur with cold acclimation and in no way do we attempt to differentiate between changes that may be adaptive to cold acclimation and those that are not. In this study, the metabolites proline, raffinose, galactinol, glucose, fructose, and sucrose increased in response to low temperature and in transgenic plants over-expressing CBF, thus providing validity to the technique and experimental design (Table 3.2). Of all the metabolites identified, approximately 70 percent changed in response to cold acclimation suggesting a global reprogramming of metabolism due to low temperature (Table 3.1 and 3.3). Due to the large numbers of metabolites that change at least 5-fold in response to low temperature we focused on the behavior of the metabolites that changed the most in response to cold acclimation. Significantly, among these 113 metabolites were glucose, fructose, proline, galactinol, sucrose, and raffinose which had all previously been show to increase to response to low temperature (Table 3.2)(Gilmour et al., 2000). Significantly 83 of these 113 metabolites were not identifiable (Table 3.2). Some of these metabolites may have cyroprotective properties. It would be of particular interest to identify these metabolites and determine if this is the case.

Previous work from Gilmour et al. has shown that over-expression of CBF mimics the accumulation of a targeted group of known metabolites that accumulate in response to low temperature (Gilmour et al., 2000). In this work we show that over-expression of CBF mimics these same changes as well as a significant proportion, 79%, of all the metabolic changes associated with cold acclimation (Table 3.5). In addition we further demonstrated that the metabolome identified by GCMS of transgenic plants over-

expressing CBF3 is more similar to cold-acclimated plants than non-acclimated plants (Figure 3.1). These observations provide further evidence that CBF may be responsible for the accumulation of these metabolites in response to low temperature; though it is possible that a metabolite increased in response to low temperature for one reason and increased in plants over-expressing CBF for another reason.

In addition, another interesting observation from this work was that CBF over-expression mimicked many of the polar metabolic changes (Table 3.5) (small polar water soluble metabolites) associated with cold acclimation but it did not influence the non-polar metabolites that changed in response to cold acclimation (Table 3.6). Metabolites, both polar and non-polar, that change in response to low temperature but do not change in plants over-expressing CBF suggest the presence of alternative signaling pathways that are responsible for the accumulation of these metabolites.

Recent transcript profiling from Fowler and Thomashow (2002) suggested the presence of other cold response pathways. These cold-regulatory pathways may be responsible for the accumulation of metabolites that are not CBF influenced. In this study we identified 80 metabolites from the GCMS (Table 3.5) and 21 metabolites from the LCMS (Table 3.6) that increase in response to cold acclimation but do not as a result of CBF over-expression. Among these metabolites are flavin based compounds, including four kaempferols and hesperidin which increased in response to low temperature (Table 3.4). These molecules are thought to serve as sunscreens due to the excess visible radiation or as antixoidants. These molecules as well as anthocyanins are synthesized via the phenylpropanoid pathway (Levya et al. 1995). Interestingly, analysis of mutants defective in flavanoid biosynthesis cold acclimate to the same level as wild-

type (Levya et al. 1995) suggesting that these molecules are not required for plants to cold acclimate; however, it does not limit the possibility that these metabolites may be important for optimal fitness at low temperature. These metabolites may be candidate metabolites under the control of these other cold response pathways. Furthermore, the fact that over-expression of CBF mimics many of the metabolic changes associated with cold acclimation does not limit the possibility of other cold regulatory networks influencing the same metabolites. This is supported by work from Xin and Browse (1998) who described a mutant, *eskimo1*, in Arabidopsis that accumulated proline, a metabolite influenced by CBF. Interestingly, no members of the CBF regulon were expressed in this mutant suggesting that the accumulation of proline may be under the control of another network independent of CBF.

To test directly the role of CBF in configuring the low temperature metabolome one would need a knockout of all the functional CBF's at low temperature. At the current time this is not available. However, recent work has shown that there is natural variation in Arabidopsis ecotypes, Ler and Cvi-1, in freezing tolerance (Martinez-Zapater et al., 2002). Genetic analysis of these ecotypes led to the identification of a QTL that maps to the CBF locus. Our work shows that this natural variation in freezing tolerance can be extended to ecotypes Ws-2 and Cvi-1 (Figure 3.3). The observation of natural variation in freezing tolerance in Arabidopsis ecotypes and that the CBF locus is a QTL which explains some of the observed variance in freezing tolerance provided a powerful tool to investigate how changes in the potency of this transcription factor affect the transcription of its target genes and changes in metabolism in response to cold acclimation.

The weak CBF locus was reflected in the accumulation of CBF transcripts in response to low temperature (Figure 3.4). In addition, we found that the levels of CBF transcript were reflected in the accumulation of CBF target (regulon) genes (Figure 3.4). There are many plausible reasons why these differences exist in the accumulation of these CBF target genes between these two ecotypes. Reasons may include but are not limited to differences in the low temperature sensing mechanism between the two ecotypes, differences in the promoters of the CBF genes between the two ecotypes, and/or differences in the proteins' ability to function correctly at low temperature. Answering which of these scenarios is most likely is a fundamental question to understanding the molecular basis of this QTL. Furthermore, we found that there was no difference in the levels of expression of a number of other cold-regulated genes (Figure 3.5) suggesting that there was not a general down regulation of all cold-regulated gene expression.

The observation that decreased CBF transcript levels were reflected in decreased levels of CBF target genes provides direct evidence that CBF is most likely primarily responsible for the induction of the CBF target genes investigated in response to low temperature. The data would suggest that there is not a compensatory mechanism that will induce the CBF target genes in response to low temperature. One possible exception is if another system exists and it also is a weak locus in Cvi and strong in Ws. Furthermore the correlation of the decreased transcript levels of P5CS and GolS (Figure 3.4) and decreased levels in the corresponding downstream metabolites provides further evidence to the role of CBF in configuring the levels of these transcripts and metabolites (Figure 3.6 and 3.7). In addition, we identified 95 metabolites that accumulate to a

greater level in Ws than Cvi (Tables 3.7 and 3.8). Significantly, a large portion, 84 percent, of these metabolites also increases in transgenic lines over-expressing CBF (Table 3.7), suggesting that CBF configures the levels of these metabolites in response to low temperature. The fact that these metabolites accumulate to greater levels in Ws may partly explain why Ws is more freezing tolerant than Cvi. Taken together these results indicate a prominent role for the CBF cold response pathway in configuring the low temperature metabolome of Arabidopsis.

Materials and Methods

Plant Growth

Arabidopsis thaliana ecotypes, Ws and Cvi, and transgenic plants constitutively over-expressing CBF3 (A28 and A30) (Gilmour et al., 2000) were used in these experiments. Seeds were stratified by placing them in water at 4°C for four days to ensure uniform germination. Following this the seeds were sown onto soil.

Arabidopsis thaliana ecotypes, Ws and Cvi, were grown in controlled environment chambers under a short day photoperiod (8 h light/16 h dark) with temperatures (20°/16°C). The illumination was 150 μmol $m^{-2}s^{-1}$. Cold Acclimation was performed for 14 days in a controlled environment chamber with a photoperiod corresponding to the photoperiod of the warm conditions at (4°/4°C). Plants were illuminated at 60-80 μmol $m^{-2}s^{-1}$.

Whole *Arabidopsis thaliana* rosettes were harvested 34 days after sowing to soil for warm samples or 30 days after sowing to soil and 14 days of cold acclimation for cold samples. 24 individual plants were harvested at two time points (dusk and dawn) in both non-acclimated and cold-acclimated samples from both ecotypes.

Arabidopsis thaliana ecotype Ws and the CBF 3 transgenic lines (A28 and A30) were grown in controlled environment chambers under a long day photoperiod (16 h light/8 h dark) with temperatures (20°/16°C). The illumination was 150 μmol $m^{-2}s^{-1}$. Whole Arabidopsis thaliana rosettes were harvested 19 days after sowing to soil (Ws) and 26 days after sowing to soil (A28 and A30). 12 individual plants were harvested from each transgenic line at dusk. Plants were grown for different periods of time as to obtain plants of the same developmental stage.

Metabolite Extraction

Samples were prepared by first homogenizing each individual plant in liquid nitrogen with a Retsch mill. Following this 25-40 mg of tissue from each sample was transferred to an eppendorf tube on liquid nitrogen for subsequent metabolite extraction.

Metabolites were extracted for GCMS using 1ml of a 1:2.5:1 chloroform:methanol:water solution for 5 minutes at 4°C. Following this 500 µl of water was added to partition against the chloroform. Samples were spun for 2 minutes at 14,000 rpm. The aqueous phase was removed. 500 µl of warm samples and 100 µl of cold and CBF over-expressing samples were lyophilized. 24 independent samples of each ecotype from both time points were analyzed for non-acclimated and cold acclimated samples. 12 independent samples of each transgenic line over-expressing CBF3 were also analyzed.

Metabolites were extracted for reverse phase LCMS from 200 mg tissue from pooled samples of 20 plants with 1.5 ml of 100% methanol for 5 minutes at 4°C. Following this samples were spun for 2 minutes at 14,000 rpm. The aqueous phase was removed and used for subsequent injection on the LCMS. 3 samples were analyzed for cold acclimated and non-acclimated samples while 2 samples were analyzed for each transgenic line over-expressing CBF3.

Metabolite Detection and Data Collection

This protocol is according to Weckwerth et al., 2004. For GC-TOF MS (Leco Pegasus II GC-TOF mass spectrometer; Leco, St. Joseph, MI, USA) analysis, the aquesous phase from the extraction was dried and dissolved in 50 µl of methoxamine

hydrochloride (20 mg/ml pyridine) and incubated at 30°C for 90 min with continuous shaking. Then 80 µl of N-methyl-N-trimethylsilyltrifluorocacetamid (MSTFA) was added to derivatize polar functional groups at 37°C for 30 min. The derivatized samples were stored at room temperature for 120 min before injection. GC-TOF analysis was performed on an HP 5890 gas chromatograph with tapered, deactivated split/splitless liners containing glasswool (Agilent, Boblingen, Germany) and 1 µl splitless injection at 230°C injector temperature. The GC was operated at constant flow of 1 ml/min helium and a 40 m 0.25 mm id 0.25 µm RTX-5 column with 10 m integrated precolumn. The temperature gradient started at 80°C, was held isocratic for 2 min, and subsequently ramped at 15°C/min to a final temperature of 330°C which was held for 6 min. Twenty spectra per second were recorded between m/z 85-500. Peak identification and quantification were performed using the Pegasus software package (Leco). Reference chromatograms were defined that had a signal/noise threshold of 20 and used for automated peak identification based on mass spectral comparison to a standard NIST 98 library. Automated assignments of unique fragment ions of each individual metabolite were taken as a default as quantifiers, and manually corrected where necessary. All artifactual peaks caused by column bleeding or phtalates were manually identified and removed from the results table. All data were normalized to plant mg fresh weight (FW) and log-transformed. A high number of replicates were used in lieu of an internal standard. All the data analysis was performed using Microsoft Excel 5.0 and Microsoft Access. The reverse phase LCMS analysis was performed according to Tolstikov et al. (2003).

Data Analysis

The metabolome of non-acclimated, cold acclimated samples, transgenic lines over-expressing CBF3 were characterized by GCMS and reverse phase LCMS. The relative quantities of a given metabolite in each sample were determined in each sample by calculating the peak area corresponding to that metabolite from the chromatogram. Peak areas have a linear relationship to changes in metabolite levels; however, changes in peak areas are not comparable between different types of metabolites due to the chemical properties of the metabolite. Each peak represented a specific metabolite and the area of that peak represented a semi-quantitative measurement of the amount of a particular metabolite. Also associated with each peak was a corresponding mass spectrum that allows for identification of the metabolite.

Once the peak area of each metabolite was determined in non-acclimated samples, cold acclimated samples, and transgenic lines over-expressing CBF3 (A28 and A30); we determined if a metabolite changed in response to cold acclimation or due to CBF over-expression by applying the following criteria. First, the metabolite had to have a peak area change greater than one (the metabolite increased) in response to cold acclimation or CBF over-expression. A change in peak area was measured by dividing the average peak area of the cold acclimated or CBF transgenic line sample by the average peak area of the non-acclimated samples. Second, the level of a given metabolite had to be significantly different in the cold acclimated samples or CBF transgenic line sample from the non-acclimated samples as measured by a p-value of less than 0.001 (GCMS) or less than 0.05 (LCMS). The p-value was calculated by a t-test comparing the cold acclimated or the CBF transgenic line sample and the non-acclimated samples. Metabolites were also

considered to change in response to cold acclimation if they were absent in the warm and present in the cold acclimated samples or transgenic lines over-expressing CBF3. Each of these criteria had to be met in each comparison for a metabolite to be considered as changing in response to cold acclimation or CBF over-expression.

Metabolites were selected as increasing to a greater level in Ws than Cvi if they met the following criteria in both comparisons. First, the metabolite was identified as being cold regulated in ecotype Ws. Second, it increased to a greater level in ecotype Ws than Cvi calculated by dividing the average peak area of each metabolite in cold acclimated Ws by the average peak area of that same metabolite in ecotype Cvi. Third, the difference in peak area between the cold acclimated samples in each ecotype was significant as measured by a p-value of <0.001 calculated by a t-test.

Hierarchical clustering was performed using Gene-spring software. Hierarchical clustering was performed of the metabolomes from non-acclimated, cold-acclimated, and transgenic plants over-expressing CBF3 identified by GCMS and reverse phase LCMS. The mean log transformed values of the peak area of each metabolite from the individual samples was calculated, and the metabolomes from each treatment were clustered using a Pearson correlation.

Whole Plant Freeze Tests

Whole Arabidopsis rosettes, ecotypes Ws and Cvi, were grown under the conditions described above. Cold acclimated and non-acclimated, were placed at -5°C in the dark for three hours followed by ice nucleation. The plants were incubated an additional 3 days at -5°. The temperature was then raised to 4°C for 12 hours to allow the

plants to thaw. The plants were removed from the freezer and placed back in the growth chamber for 5 days to recover. The plants were then scored for freezing damage.

RNA Gel Blot Hybridization Analysis

Arabidopsis thaliana ecotypes Ws and Cvi were grown on Gamborg's B5 medium as described by Gilmour et al. (1998) at 24°C instead of 22°C. Plants were cold treated as described by Fowler and Thomashow (2002). Total RNA was extracted from Arabidopsis plants using the RNeasy plant mini kit (Qiagen Inc., Valencia, CA) as detailed by the manufacturer. Northern transfers were prepared and hybridized as described by Hajela et al. (1990) and washed at high stringency as described by Stockinger et al. (1997). Full length cDNAs were labeled with P^{32} using the Random Primers DNA Labeling System (Invitrogen Life Technologies Carlsbad, CA) as directed by the manufacturer.

Expression levels were quantified by normalizing to an rRNA probe to account for loading and transfer differences. Expression levels were quantified using the Quantity One program from Biorad. Expression levels were then expressed as a percentage of the highest point of accumulation (100%) for each specific transcript to indicate relative expression levels.

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Chapter 4

The Circadian Clock Gates the Levels of *CBF* Transcripts in Response to Low Temperature

Summary

The Arabidopsis CBF cold response pathway has a central role in cold acclimation, the process whereby plants increase their freezing tolerance in response to low nonfreezing temperatures. Recently it has been shown that basal levels of *CBF* and some CBF target genes exhibit circadian cycling. This raised the question if plants transferred to low temperatures at different points in a circadian rhythm would show differences in the cold regulated gene expression of *CBF*. The results indicate that *CBF* expression levels in response to low temperature showed a gating response due the circadian clock. The highest levels of *CBF* expression corresponded to 4 hours after subjective dawn and the lowest levels of *CBF* expression corresponded to 16 hours after subjective dawn with a periodicity of 24 hours. Recently it was proposed that light was required for the induction of the CRT/DRE *cis*-acting element. To investigate this observation more thoroughly we examined how light at low temperature influenced expression of the CBF cold response pathway. We found that light was not required for the induction of the CRT/DRE *cis*-acting element. Furthermore we found that light was not required for the maximum induction of CBF.

Introduction

Many plants have the ability to sense low temperature and respond by activating mechanisms that lead to an increase in freezing tolerance. This adaptive response is known as cold acclimation. At present, the best understood genetic system that has a role in cold acclimation is the Arabidopsis CBF cold response pathway. Exposing Arabidopsis plants to low temperature results in rapid induction of a small family of transcriptional activators known either as *CBF1*, *2* and *3* (Gilmour et al., 1998; Medina et al., 1999) or *DREB1b*, *c* and *a*, respectively (Liu et al., 1998). These transcription factors, which belong to the AP2 domain family of DNA binding proteins, recognize a *cis*-acting regulatory element known as the CRT (C-repeat)/DRE (dehydration response element) (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994) that is present in the promoters of many cold-inducible genes such as *COR15a* and *COR78* (also known as *RD29A* and *LTI78*) (Stockinger et al., 1997). Transgenic plants over-expressing CBF result in constitutive expression of the COR genes and freezing tolerance that is independent of a cold stimulus (Jaglo-Ottosen et al., 1998).

Low temperature and light are known to entrain the circadian clock (Kreps and Simon, 1997). Interestingly, recent studies have shown that the transcript levels for *CBF3* and target COR genes exhibit circadian-regulated cycling at warm temperatures in addition to being induced by low temperature (Harmer et al., 2000; Gilmour et al., 1998). These results are quite intriguing, first because the chilling tolerance of plants is known to cycle in a circadian fashion (Rikin et al., 1993) and second because low temperature is known to entrain the circadian clock (Heintzen et al., 1994; Kreps and Simon, 1997).

Furthermore, recent studies have shown that light influences cold acclimation and cold regulated gene expression. In one study it was demonstrated that light was required for the full development of cold acclimation (Wanner and Junttila, 1999). More recently it was suggested that light and light signaling influenced expression levels of the CBF cold response pathway in response to low temperature. Kim et al. (2002) reported that light was required for the induction of the CRT/DRE *cis*-acting element in response to low temperature. In addition, they reported that light enhanced the induction of *CBF* in response to low temperature. Furthermore they demonstrated that phytochrome B was necessary for the induction of the CRT/DRE in response to low temperature. We found these results to be rather interesting (not intuitive) since plants can experience low temperature in both light conditions and dark conditions.

We were very interested in exploring the observations described above. First we investigated if the rhythms entrained by the circadian clock influenced the cold regulated gene expression of *CBF* and other cold regulated genes. Our results demonstrate that the circadian clock gates the level of *CBF* in response to low temperature with the highest levels of *CBF* induction in response to low temperature at 4 hours after subjective dawn and the lowest levels of *CBF* induction 16 hours after subjective dawn. In addition, our results show that the circadian clock gates the cold regulated gene expression of one transcription factor, *Rav1*; but not *ZAT12* both of which are coordinately regulated like *CBF* in response to low temperature. Interestingly, the peaks and troughs of basal *CBF* and *RAV1* levels corresponded to the peaks and troughs of *CBF* and *RAV1* levels in response to low temperature. Second we investigated how light influenced cold regulated gene expression. We found that light was not required for induction of the CRT/DRE in

response to low temperature. In addition we found that CBF levels were not altered significantly be light. Furthermore, we found that CBF levels were not altered in the phytochrome or cryptochrome mutants.

Results

The Circadian Clock "Gates" *CBF* expression levels in response to low temperature

Work by Harmer et al. (2000) had indicated that transcript levels for *CBF3* and target COR genes exhibit circadian-regulated cycling at warm (non-cold) temperatures (i.e. when not induced by low temperature). This raised the question if plants transferred to low temperature at different points in a circadian rhythm would have any effect on the extent of *CBF* induction by low temperature.

To begin to investigate this question, we transferred plants grown in a photoperiod (12:12) into continuous light, at 4°C at 6 hours intervals for 48h. Samples were harvested before transfer to cold and then after 1, 4, 8, and 24 h at 4°C. *CBF* levels were then determined by northern analysis. Results indicated that the maximal level of *CBF* induction in response to 4°C was at 4 hours after subjective dawn (generally after 1-4 h at 4°C) and cycled with a period of approximately 24 h (Figure 4.1). In contrast, the lowest levels of cold induction corresponded to 16 h after dawn with a period of approximately 24 h (Figure 4.1) These observations suggest that the circadian clock may be "gating" the response of CBF transcript levels to low temperature.

Figure 4.1

The Circadian Clock Gates *CBF* Expression Levels in Response to Low Temperature

Northern blot hybridization showing *CBF* expression levels in response to low temperature. Arabidopsis, ecotype Columbia, was gown on a 12:12 photoperiod and released into free running conditions. Following this they were transferred to low temperature for the time indicated at the circadian times listed (period=6 h).

For the graphical data, transcript induction is normalized using *EIF4a* expression levels, relative to the 0 h time point at circadian time 4

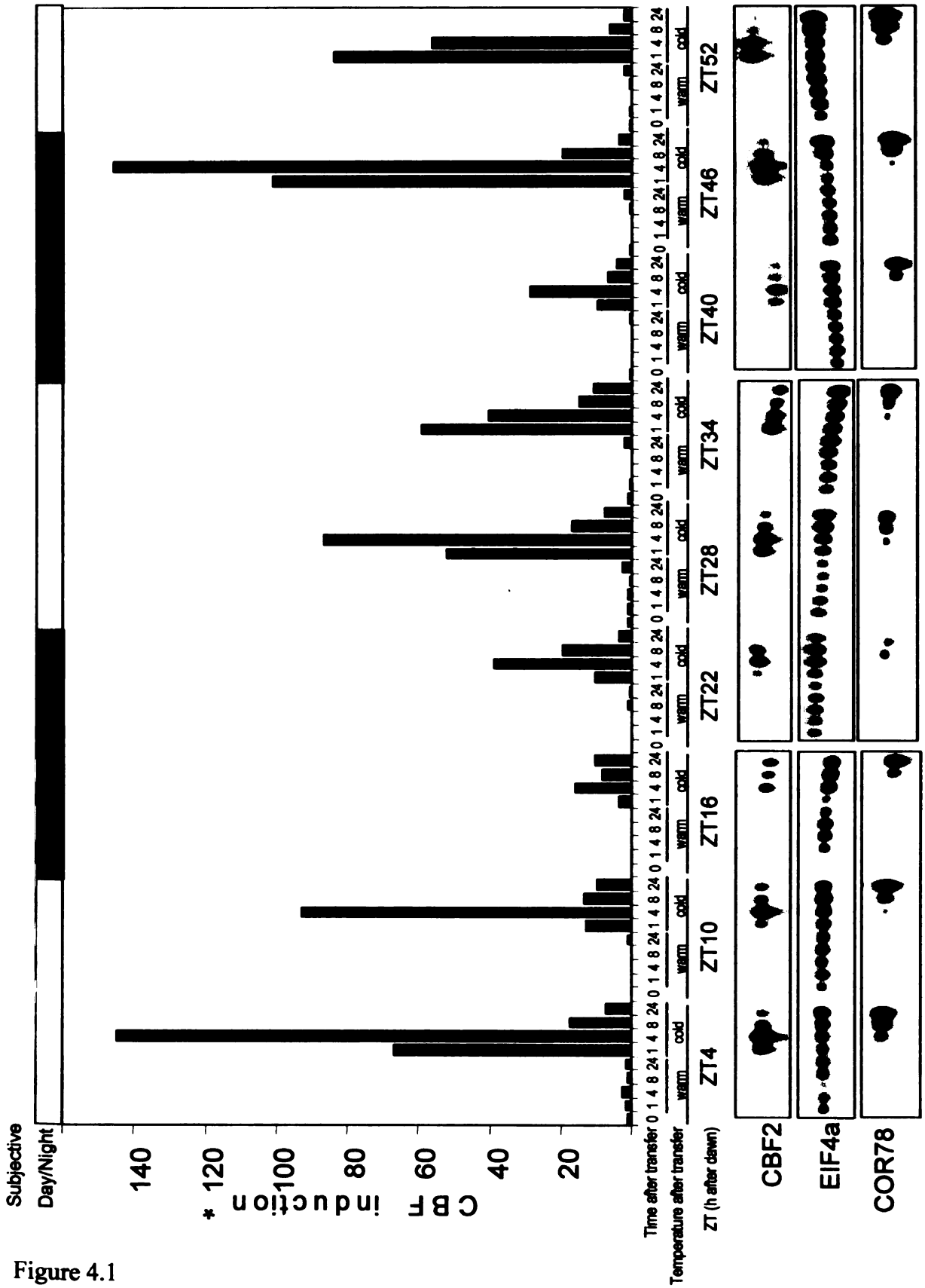

Figure 4.1

The Circadian Clock Gates *CBF* Expression Levels in Response to Low Temperature

The Circadian Clock Gates the Response of *CBF* transcript levels to low temperature

To determine whether the circadian clock is gating *CBF* transcript levels in response to low temperature, we examined *CBF* transcript levels in transgenic plants in which circadian cycling had been abolished. Circadian Clock Associated 1 (CCA1) is thought to be a component of the central circadian oscillator and transgenic plants constitutively expressing CCA1 exhibit arrhythmicity of all tested circadian rhythms (Schaffer et al, 1998, Wang and Tobin, 1998). CCA1 plants were therefore an ideal tool to test whether the cycling of maximal *CBF* induction by low temperature requires a functional circadian clock.

CCA-1 transgenic plants were grown in a photoperiod (12:12) in parallel with wild-type plants from figure 4.1, released into continuous light, then transferred to 4°C. Samples were harvested after 1, 4, and 24 h at low temperature. Northern blot analysis was used to examine induction of *CBF* transcript levels in response to low temperature. Cycling of maximal *CBF* induction in response to low temperature was abolished in the CCA1 over-expressing transgenic plants indicating that a functional circadian clock was required to gate the low-temperature response of *CBF* expression (Figure 4.2).

An alternative explanation to the above conclusion is that the circadian clock may be gating the response of *CBF* transcript levels to decreased light levels. This possibility exists because when the plants were transferred to 4°C, the light levels were also decreased in order to minimize photo-oxidative stress due to the combination of high light and low temperature.

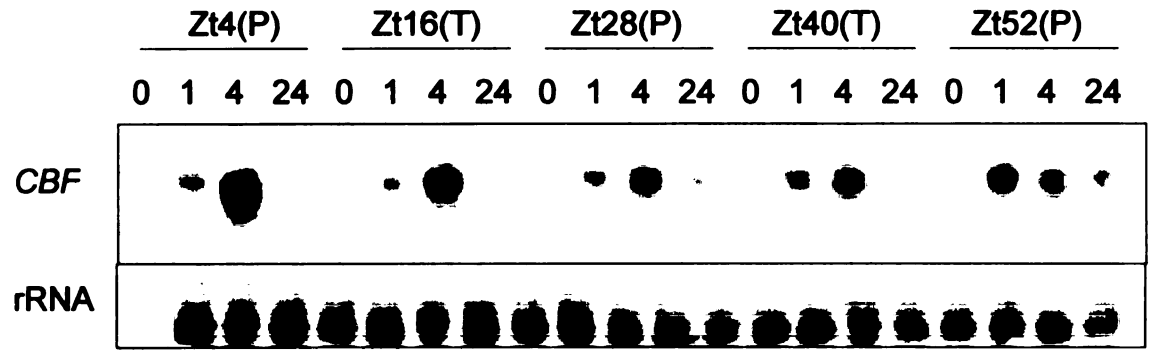

Figure 4.2

Transgenic plants over-expressing CCA-1 abolish the gating of *CBF* expression levels in response to low temperature by the circadian clock.

Northern blot analysis showing *CBF* expression levels in response to low temperature. Transgenic plants over-expressing CCA-1 were gown on a 12:12 photoperiod and released into free running conditions. Following this they were transferred to low temperature for the time indicated at the circadian times listed (period=12 h). rRNA was used as a loading control.

To determine whether the circadian clock rather than decreased light levels was gating *CBF* induction in response to low temperature we examined *CBF* induction in plants grown and cold treated in similar light levels. Plants were either grown in "high light" conditions (70-80 μmol $m^{-2}s^{-1}$) or "low light" conditions (15-25 μmol $m^{-2}s^{-1}$) then transferred to 4°C with the same light levels. In both cases, cycling of maximal *CBF* induction after transfer to 4°C was observed (data not shown) indicating that the circadian clock rather than decreased light levels was gating the low temperature response.

COR genes and the CRT/DRE are not gated by the circadian clock in response to low temperature

The CBF family of transcriptional activators regulates the expression of a large set of cold-responsive genes (the CBF regulon) (Fowler and Thomashow, 2001). We expected that the peak induction levels of transcripts for CBF target genes in response to cold would mirror the cycling pattern for CBF itself. To test this we examined the low temperature induction of transcript levels for the CBF-target gene, *COR 78*. Surprisingly, the maximal induction of *COR 78* transcipt by cold did not follow a 24 h cycle and was essentially arrhythmic (Figure 4.1). Similar results were also seen for *COR 15a* (data not shown).

This result suggested that other regulatory factors might be compensating for the lower *CBF* levels at the trough, in activating transcription of *COR78*. We investigated this possibility using a transgenic line expressing the *GUS* reporter gene under the control of a 180 bp fragment of the *COR15a* promoter containing two CRT/DRE elements. In this transgenic line, activation of the CRT/DRE by CBF should determine the expression

level of the *GUS* transcript. As described above, these plants were grown in a 12:12 photoperiod, released into continuous light, then transferred to 4°C, and samples harvested. Similar to *COR78* transcript levels, the levels of the *GUS* transcript did not reflect the cycling of maximal *CBF* expression levels (Figure 4.3). This suggests that the fact that maximal induction of *COR78* transcript levels by cold do not cycle in parallel with CBF and may not be due to the compensatory activity of another transcription factor(s) binding elsewhere in the *COR78* promoter. This result may indicate that the amount of transcript produced at the trough of *CBF* induction is sufficient for maximal induction of the target genes via the CRT/DRE.

Rav1 is gated by the circadian clock in response to low temperature but _Zat12_ is not

Fowler and Thomashow (2001) recently identified two transcription factors, *Zat12* and *Rav1*, that are cold-regulated in parallel with *CBF* in response to low temperature. *CBF*, *RAV1*, and *ZAT12* show similar expression patterns in response to other stimuli (JV and DGZ unpublished results). It was therefore of interest to determine whether the induction of *RAV1* and/or *ZAT12* transcript levels by cold was also gated by the circadian clock in a similar fashion to *CBF*.

Northern blot analysis of *RAV1* and *ZAT12* (using the RNA samples analyzed in figure 4.1) revealed that the maximal levels of *RAV1* transcript cycled in a manner similar to *CBF* while the maximal levels of *ZAT12* were essentially arrhythmic. Thus cold regulated induction of *RAV1* by cold is gated by the circadian clock while induction of *ZAT12* transcript is not (Figure 4.4). Interestingly, Harmer et al (2000) have shown that basal *RAV1* expression is circadian-regulated. However, their data did not identify

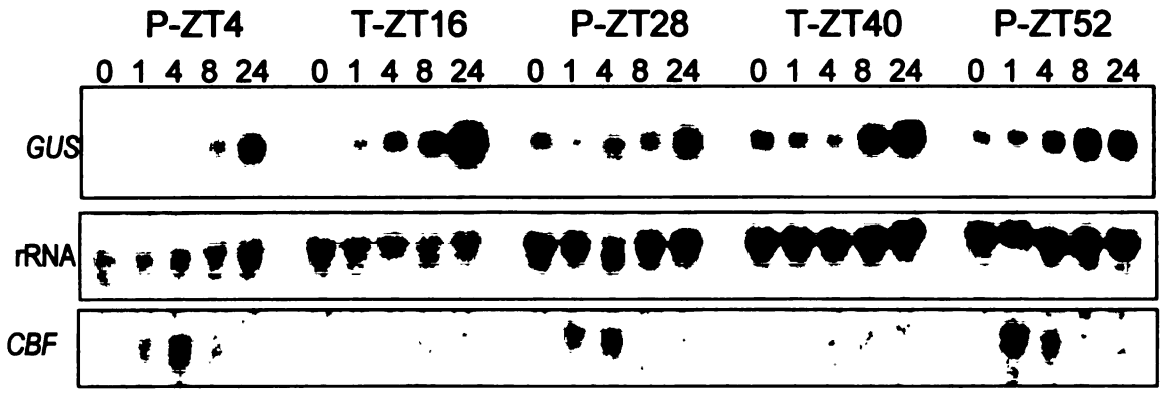

Figure 4.3

The CRT/DRE:*GUS* is not gated by the circadian clock in response to low temperature

Northern blot analysis showing *GUS* and *CBF* expression levels in response to low temperature in transgenic plants with a CRT/DRE fragment driving *GUS*. Transgenic plants were gown on a 12:12 photoperiod and released into free running conditions. Following this they were transferred to low temperature for the time indicated at the circadian times listed (period=12 h). rRNA was used as a loading control.

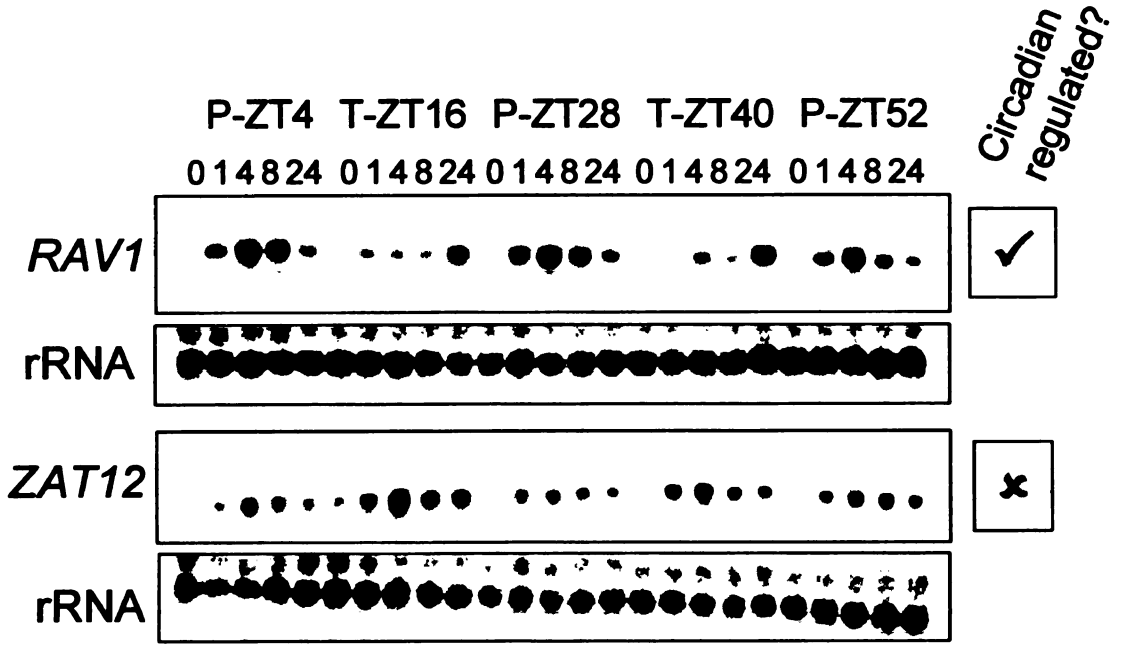

Figure 4.4

RAV1 is gated by the circadian clock in response to low temperature while *ZAT12* is not.

Northern blot analysis showing *RAV1* and *ZAT12* expression levels in response to low temperature in wild type Arabidopsis. Plants were gown on a 12:12 photoperiod and released into free running conditions. Following this they were transferred to low temperature for the time indicated at the circadian times listed (period=12 h). rRNA was used as a loading control.

ZAT12 as exhibiting circadian regulation. Furthermore the phase of circadian cycling of *RAV1* was the same as *CBF*, with a peak of ZT4 and a trough of ZT16.

Light is not required for the induction of *CBF*

To begin to investigate how light influence cold regulated gene expression, plants were grown in a 12:12 photoperiod and transferred 4 h after dawn to complete darkness or continuous light at 4°C. Plants were harvested after 1h, 4h, 8h, and 24h. Northern blot analysis was performed to evaluate the induction of CBF. We observed increased transcript levels of *CBF* in the plants transferred to the dark when compared to the light (Figure 4.5). These results suggest that light is not required for the induction of *CBF*.

The CRT/DRE does not require light for induction

Furthermore we investigated if the CRT/DRE *cis*-acting element required light for its induction. We investigated this using a transgenic line expressing the *GUS* reporter gene under the control of a 180 bp fragment of the *COR15a* promoter containing two CRT/DRE elements. In this transgenic line, activation of the CRT/DRE by CBF should determine the expression level of the *GUS* transcript. As described above, these plants were grown in a 12:12 photoperiod, transferred to 4°C at 4 h after dawn to complete darkness or continuous light, and then samples were harvested. Northern blot analysis was performed to evaluate *GUS* expression. The analysis revealed similar levels of *GUS* in response to cold in the dark or the light (Figure 4.5). These results suggest that light is

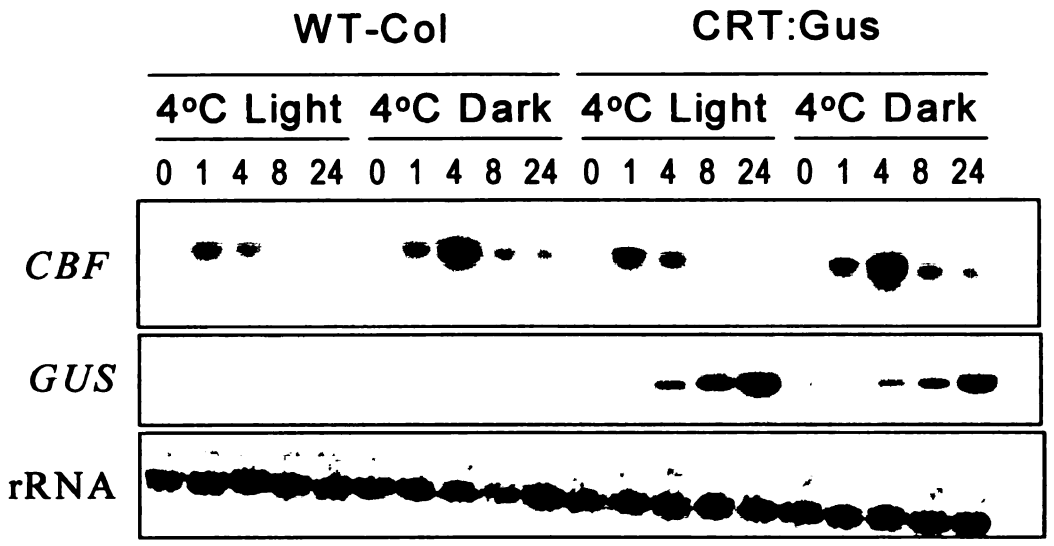

Figure 4.5

CBF and CRT/DRE:*GUS* expression levels in response to low temperature in the presence or absence of light

Northern blot analysis showing *CBF* expression levels in response to low temperature are similar in the absence or presence of light. It also shows expression levels of a minimal promoter of the CRT/DRE:*GUS* in response to low temperature are similar in the absence of presence of light. Col is the Columbia ecotype of Arabidopsis and the CRT/DRE:*GUS* is a transgenic line with this minimal promoter. Plants grown on a 12:12 photoperiod were transferred to 4°C for the indicated times at circadian time 4 into darkness or continuous light. rRNA was used as a loading control.

not required for the induction of the CRT/DRE and that the weaker induction in the light may be compensated.

The phytochrome or cryptochrome photoreceptors are not required for *CBF* induction

To further explore the influence of light or its signaling pathways on cold regulated gene expression we examined *CBF* expression levels in *phyA*, *phyB*, *phyA/B*, and *cry1/2* mutant backgrounds. Plants were grown in a 12:12 photoperiod, transferred to 4°C at 4 h after dawn to continuous light, and then samples were harvested. Northern Blot analysis showed that the induction of *CBF* in response to low temperature was not affected in any of these mutants (Figure 4.6). These results would suggest that the photoreceptors are not necessary for the proper induction of the *CBF* under the conditions tested.

Discussion

Central to our understanding of cold acclimation is identifying the genes that are cold responsive, determining their role at low temperature, and determining how the expression of these genes are modulated. Recent studies have identified a number of genes that are responsive to low temperature (Fowler and Thomashow, 2002; Seki et al., 2000) while others have focused on identifying upstream proteins that influence their regulation (Zhu and colleagues, 1996). In this study we investigated how the circadian clock, an endogeneous timekeeper that is entrained by light and low temperature, may

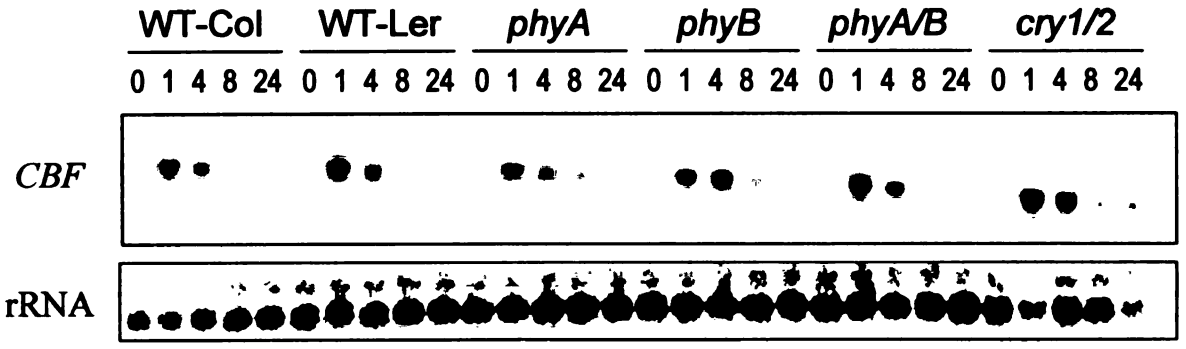

Figure 4.6

CBF expression levels in response to low temperature in Wild type, phytochrome mutants, and cryptochrome mutants.

Northern blot analysis showing *CBF* expression levels in response to low temperature are similar to wild-type in the phytochrome and cryptochrome mutants. Col is the Columbia ecotype and Ler is the Landsberg erecta ecotype of Arabidopsis. The photoreceptor mutants analyzed were the *phyA*, *phyB*, *phyA/B*, *cry1/2*. Plants grown on a 12:12 photoperiod were transferred to 4°C for the indicated times at circadian time 4 into continuous light. rRNA was used as a loading control.

influence cold-regulated gene expression how an external environmental factor such as light influences cold-regulated gene expression.

Recent work from Zarka et al. (2003), has shown that an external environmental factor such as the absolute temperature may modulate levels of *CBF* and COR gene expression. First, we show that expression levels of *CBF* in response to low temperature are modulated by the circadian rhythms at which plants are transferred to the cold (Figure 4.1). This phenomena has been described as gating by the circadian clock, whereby the response to an external stimulus such as light or low temperature varies with the time of day (Carre, 2002). Interestingly, *CBF* target genes such as the COR genes were not reflective of the levels of CBF transcription (Figure 4.1). In addition, we were able to show that the expression levels of *RAV1* in response to low temperature, a gene that is coordinately regulated with *CBF*, are modulated by the circadian rhythms at which point the plants are transferred to the cold (Figure 4.4). Furthermore we found that *ZAT12* another transcription factor that is coordinately regulated with *CBF* and *RAV1* in response to low temperature does not showing the gating phenomena by the circadian clock (Figure 4.4).

From our results we may conclude that the gating phenomena by the circadian clock in response to low temperature appears to require first, that the gene shows circadian regulated cycling and second, that the gene is immediate downstream of the low temperature stimulus. Both of these requirements are met in *CBF* and *RAV1*, two genes which show the gating phenomenon by the circadian clock in response to low temperature. However in the case of *ZAT12* and the COR genes both requirements are not fulfilled. In the case of *ZAT12*, it is induced immediately downstream of the low

temperature stimulus but it does not show circadian regulated cycling of its basal levels (Harmer et al., 2000). And in the case of *COR15a* and *COR78*, both genes show circadian regulated cycling but they are not immediately downstream of the low temperature stimulus.

So, what may be causing the gating phenomena by the circadian clock? From the results one may speculate that some factor exists between the low temperature sensor and the induction of *CBF* genes that is modulated by the circadian clock. Another possible connection is the correlation between the peaks and troughs of basal CBF expression and the peaks and troughs of *CBF* expression in response to low temperature. One possible explanation is calcium. Interestingly, calcium levels also exhibit circadian regulated cycling (Johnson et al., 1995). Calcium has also been implicated in the expression of cold regulated genes (Knight et al., 1996), although no direct evidence has been shown for its involvement in *CBF* expression levels in response to low temperature. Calcium peaks just after subjective dawn and *CBF* peaks about 2-4 hours later. Possibly as calcium levels cycle due to the circadian clock it directly influences the transcript accumulation of *CBF* or it modulates some component that influences *CBF* expression.

Second, we investigated if light or light signaling pathways influenced CBF expression and the CRT/DRE *cis*-acting element. The data from our experiments would suggest that the light or its signaling pathways are not required for induction of the CRT/DRE *cis*-acting element (Figure 4.5, 4.6). In addition our results show that light is not required for the induction of *CBF* in response to low temperature (Figure 4.5). These results are in contrast to the results from Kim et al. (2000). They proposed that light and its signaling pathways via PHYB were required for induction of the CRT/DRE. In

addition they proposed that light was required for full induction of *CBF* gene expression in response to low temperature. The results from our study we find to be more intuitive since plants may experience periods of low temperature at night or during the light.

The fact that the results from the two labs are contrary to each other is rather interesting. One potential reason is that the CRT/DRE:*GUS* construct used by Kim et al. (2002) was different than the one used in these studies. This may have potentially caused some difference in observations. Also northern blots were used to evaluate gene expression analysis in response to low temperature while Kim et al. (2002) used RT-PCR to evaluate gene expression.

Methods

Plant Material and Growth Conditions (including description of transgenic plants)

Arabidopsis was grown on Gamborg's B5 medium as described by Gilmour et al. (1998) at 24°C instead of 22°C and on a 12:12 photoperiod. Plants were transferred to low temperature as described by Fowler and Thomashow (2002) at the circadian times indicated in the text of the paper. Arabidopsis ecotype Columbia was used for all the described experiments except for the transgenic lines. Transgenic lines used were plants ectopically over-expressing CCA-1, a CRT/DRE fragment driving *GUS*, and a 155 bp dimer fragment from the *CBF 2* promoter driving *GUS*.

RNA Gel Blot Hybridization Analysis

Total RNA was extracted from Arabidopsis plants using the RNeasy plant mini kit (Qiagen Inc., Valencia, CA) as detailed by the manufacturer. Northern transfers were

prepared and hybridized as described by Hajela et al. (1990) and washed at high stringency as described by Stockinger et al. (1997). Full length cDNAs were labeled with P^{32} using the Random Primers DNA Labeling System (Invitrogen Life Technologies Carlsbad, CA) as directed by the manufacturer.

Expression levels were quantifed by normalizing to an rRNA probe. Expression levels were quantified using the Quantity One program from Biorad. Levels of accumulation were then expressed as a function of fold change relative to the 0 time point at circadian time 4 for Figure 4.1.

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Chapter 5

Future Directions

This thesis investigated a number of questions surrounding the subject of molecular and metabolic responses to dehydration and cold acclimation. This research was able to advance each area investigated as it related to responses of plants to the abiotic environment. As with any research topic answering a set of questions usually results in many more future ideas to investigate. Here a brief overview of the conclusions from each chapter will be presented along with questions to be further investigated as it relates to each chapter of the thesis.

In the second chapter CBF4 was identified, a transcriptional regulator of drought responses in Arabidopsis. The data showed that over-expression of CBF4 resulted in constitutive expression of the COR genes without a stimulus and resulted in plants that are more freezing and drought tolerant. Furthermore, the data showed that *CBF4* was responsive to drought and ABA but not low temperature.

Some further areas of investigation in regard to conclusions from chapter 2 are the following. First, it may be informative to identify the regulon of genes activated by CBF4 and determine if these are the same as those activated by CBF1, 2, and 3. Next, the accumulation of the CBF4 protein may be investigated to determine if its mirrors transcript induction. Furthermore, the *cis*-acting element(s) in the promoter of *CBF4* that are responsible for its induction may be identified through promoter deletion type

experiments. In addition, an approach similar to Zhu and colleagues may be used by fusing the promoter of *CBF4* to luciferase to identify upstream regulators of *CBF4*.

In the third chapter the first global analysis of the metabolic changes associated with cold acclimation and the role of CBF in configuring these changes was investigated. The data showed that a large number of metabolic changes were associated with cold acclimation and that CBF was responsible for configuring many of these changes. The data supporting the relative importance of CBF was supported by transgenic plants over-expressing CBF and Arabidopsis ecotype Cvi which has a weak CBF locus.

Some ideas to investigate further from Chapter 3 are the following. First, the unidentified metabolites that change to significant levels in response to low temperature and that are controlled by CBF over-expression may be identified. Second, it would be interesting to identify the metabolic changes in transgenic plants over-expressing other transcription factors that are induced in response to low temperature. In addition, the metabolic changes associated with cold acclimation may be investigated in mutants that exhibit a constitutively cold acclimated phenotype such as *eskimo 1* and mutants that are sensitive to freezing such as *sfr6*. Furthermore a screen could be initiated to identify mutants altered in the accumulation of a targeted group of metabolites, those that accumulate the most in response to low temperature. Research could also be initiated to study the global molecular/genetic responses to low temperature in ecotype Cvi which is sensitive to freezing temperatures and ecotype Ws which is not sensitive to freezing temperatures. Next, the molecular factors responsible for the weak CBF locus in Cvi may be identified.

In the fourth chapter the results showed that the circadian rhythms entrained by the circadian clock influence cold regulated gene expression. The data shows that the circadian clock gates the levels of CBF transcripts in response to low temperature. The data also showed that Rav1 expression levels were also gated by the circadian clock in response to low temperature, but the transcript levels of two other genes, Cor78 and Zat12, were not gated by the circadian clock.

From the fourth chapter it would be insightful to investigate the following. First it would be informative to do a global analysis of genes whose levels are gated in response to low temperature. Next one could compare these genes to those that show basal circadian cycling. This could be used to investigate the hypothesis that the gating phenomena requires first that the gene is circadian regulated and second that its induction is immediately downstream of the low temperature stimulus. Furthermore one could investigate if the gating phenomena of CBF expression levels by the circadian clock can be extended to other stimuli that induce CBF expression such as mechanical agitation.

Research continues to progress at a rapid pace in answering important scientific questions. This thesis contributed to three areas related to how plants respond to low temperature and dehydration. From this thesis a number of other scientific questions have arisen of significant importance that are available for further study.