Cold Induction of Arabidopsis *CBF* Genes Involves Multiple ICE (Inducer of *CBF* Expression) Promoter Elements and a Cold-Regulatory Circuit That Is Desensitized by Low Temperature[1]

Daniel G. Zarka, Jonathan T. Vogel, Daniel Cook, and Michael F. Thomashow*

Michigan State University-Department of Energy Plant Research Laboratory (D.G.Z., J.T.V., D.C., M.F.T.) and Department of Crop and Soil Sciences (M.F.T.), Michigan State University, East Lansing, Michigan 48824

The Arabidopsis *CBF1*, *2*, and *3* genes (also known as *DREB1b*, *c*, and *a*, respectively) encode transcriptional activators that have a central role in cold tolerance. *CBF1-3* are rapidly induced upon exposing plants to low temperature, followed by expression of CBF-targeted genes, the CBF regulon, resulting in an increase in plant freezing tolerance. At present, little is known about the cold-sensing mechanism that controls *CBF* expression. Results presented here indicate that this mechanism does not require a cold shock to bring about the accumulation of *CBF* transcripts, but instead, absolute temperature is monitored with a greater degree of input, i.e. lower temperature, resulting in a greater output, i.e. higher levels of *CBF* transcripts. Temperature-shift experiments also indicate that the cold-sensing mechanism becomes desensitized to a given low temperature, such as 4°C, and that resensitization to that temperature requires between 8 and 24 h at warm temperature. Gene fusion experiments identified a 125-bp section of the *CBF2* promoter that is sufficient to impart cold-responsive gene expression. Mutational analysis of this cold-responsive region identified two promoter segments that work in concert to impart robust cold-regulated gene expression. These sequences, designated ICEr1 and ICEr2 (induction of CBF expression region 1 or 2), were also shown to stimulate transcription in response to mechanical agitation and the protein synthesis inhibitor, cycloheximide.

Many plants increase in freezing tolerance in response to low nonfreezing temperatures, a phenomenon known as cold acclimation (Guy, 1990; Thomashow, 1999). In Arabidopsis, cold acclimation involves action of the CBF cold-response pathway (Thomashow, 2001). Within 15 min of exposing plants to low temperatures, transcripts accumulate for a family of genes designated *CBF1*, *CBF2*, and *CBF3* (Gilmour et al., 1998; Jaglo-Ottosen et al., 1998; Medina et al., 1999), or *DREB1b*, *DREB1c*, and *DREB1a* (Liu et al., 1998), respectively, which encode transcriptional activators that are members of the AP2/EREBP family of DNA-binding proteins (Riechmann and Meyerowitz, 1998). These transcription factors bind the cold- and dehydration-responsive DNA regulatory element designated the CRT (C-repeat)/DRE (dehydration response element); (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger et al., 1997) that is present in the promoters of *COR* and many other cold-responsive genes and stimulate their transcription. Expression of the CBF regulon of target genes then leads to an increase in freezing tolerance (Gilmour et al., 1998; Jaglo-Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999). Multiple mechanisms appear to contribute to the enhancement of freezing tolerance, including the synthesis of cryoprotective polypeptides, such as COR15a (Artus et al., 1996; Steponkus et al., 1998), and the accumulation of compatible solutes that have cryoprotective properties, including Suc, raffinose, and Pro (Nanjo et al., 1999; Gilmour et al., 2000; Taji et al., 2002).

Currently, little is known about how the *CBF* genes are up-regulated in response to low temperatures, but important insights are beginning to emerge. It has been established that the promoters of the *CBF* genes are responsive to low temperatures (Shinwari et al., 1998), and a transcription factor, Inducer of CBF Expression 1 (ICE1) that has a role in *CBF* expression has recently been identified (Chinnusamy et al., 2003). ICE1 encodes a MYC-like basic helix-loop-helix (bHLH) protein. A dominant-negative mutation of *ICE1*, *ice1*, results in almost complete elimination of *CBF3* transcript accumulation in response to low temperatures. Significantly, however, the *ice1* mutation has little effect on cold-induced accumulation of *CBF2* transcripts, indicating that there are differences in mechanisms of expression within the CBF/DREB1 gene family. Other genes and proteins that affect *CBF* expression have also been identified. *LOS4* encodes a

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* Corresponding author; e-mail thomash6@msu.edu; fax 517–353–9168.

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DEAD-box RNA helicase that appears to have a positive role in *CBF* expression; accumulation of *CBF* transcripts is reduced or delayed in plants that are homozygous for the recessive *los4*-1 mutant allele (Gong et al., 2002). In contrast, *FRY2* (Xiong et al., 2002) and *HOS1* (Lee et al., 2001) appear to downregulate *CBF* expression. The FRY2 and HOS1 proteins are, respectively, a novel transcriptional repressor and a novel RING finger protein. It has been suggested that HOS1 may be an E3 ligase that targets CBF regulatory proteins for ubiquitination and protein degradation (Lee et al., 2001).

In this study, we further examine factors that affect the accumulation of *CBF* transcripts in response to low temperatures. The results indicate that steady-state levels of *CBF* transcripts increase in response to cold shock and gradual temperature downshifts; that the cold-sensing mechanism becomes desensitized with time at low temperature; that the *CBF* transcripts have a very short half-life at warm temperature; and that multiple promoter *cis*-acting regulatory elements function together to stimulate *CBF2* transcription in response to low temperature.

RESULTS

CBF Transcripts Accumulate in Response to Cold Shock and a Gradual Decrease in Temperature

Transferring Arabidopsis plants abruptly from 20°C to 4°C results in the rapid accumulation of *CBF* transcripts (Fig. 1A; Gilmour et al., 1998; Liu et al., 1998; Medina et al., 1999). In our experiments, *CBF* transcript levels reached a maximum at about 3 h and then declined significantly, but remained elevated over those found in warm-grown plants over the course of the 3-week experiment. The magnitude of the cold shock affected the peak levels of the *CBF* transcripts (Fig. 1B). That is, when plants were transferred from 20°C to 10°C, the *CBF* levels after 2 h were less than if the plants were transferred from 20°C to 4°C. Similarly, higher levels of *CBF* transcripts were observed when the cold shock was from 20°C to −5°C compared with the 20°C to 4°C treatment.

To determine whether the accumulation of *CBF* transcripts was dependent upon a rapid cold shock, plants were slowly cooled at a rate of 2°C h^{-1}, and *CBF* transcript levels were determined at various times (Fig. 1C). The results indicated that a gradual drop in temperature from 20°C to 4°C (over an 8-h time period) resulted in *CBF* levels that were essentially the same as those obtained with an abrupt 20°C to 4°C cold-shock treatment (Fig. 1C). The threshold temperature at which accumulation of *CBF* transcripts became detectable was 14°C. As temperatures continued to drop, the levels of *CBF* transcripts continued to increase as did the transcript levels of the *CBF* target gene, *COR15a*. Taken together, the results of the cold shock and gradual temperature downshift experiments indicated that the cold-sensing mechanism is not a "binary" on and off system, but instead, consists of a circuit that can monitor absolute temperature and act like a rheostat to adjust the output—the level of *CBF* transcript accumulation—to the level of low temperature input.

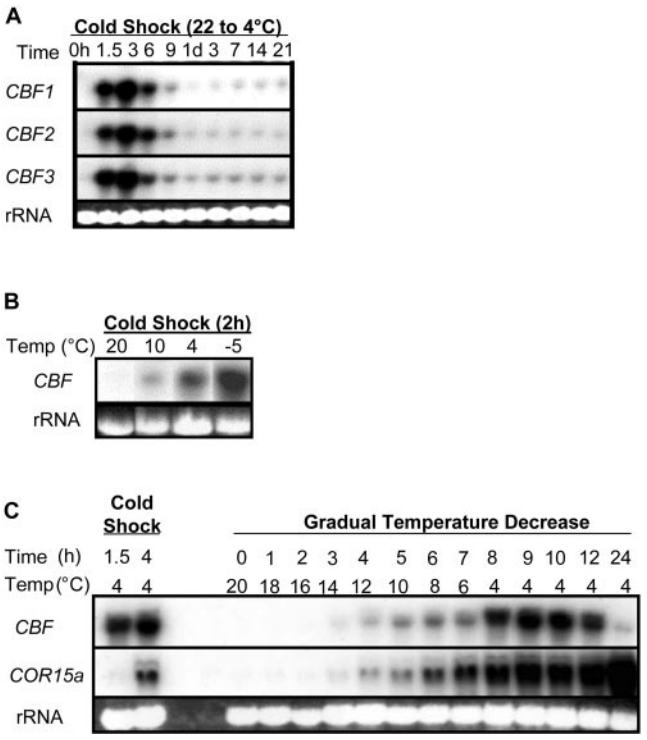

Figure 1. Accumulation of *CBF* transcripts in response to low temperatures. A, Plants were abruptly transferred from 22°C to 4°C for the indicated periods and *CBF* transcript levels were determined by RNA-blot hybridization. Gene-specific probes were used to detect, independently, *CBF1*, *2*, and *3* transcripts. B, Plants were abruptly transferred from 22°C to the indicated cold temperatures for 2 h and *CBF* transcript levels were determined by RNA-blot hybridization. The probe used, a full-length cDNA of CBF1, cross-hybridized with *CBF1*, *2*, and *3* transcripts. C, Plants grown at warm temperatures were subjected to a cold shock or to a gradual temperature decrease, and *CBF* and *COR15a* transcript levels were determined by RNA-blot hybridization. The full-length cDNA for CBF1 was used to detect *CBF1*, *2*, and *3* transcripts. In each experiment, rRNA stained with ethidium bromide was used to compare loading.

CBF Induction Involves a Cold-Sensing Mechanism That Becomes Desensitized with Time at Low Temperatures

In the cold-shock and gradual temperature downshift experiments, the levels of *CBF* transcripts decreased upon continued exposure of the plants to low temperatures. To investigate this phenomenon further, plants were moved back and forth between 20°C and 4°C at 90-min intervals and the levels of *CBF* transcripts were determined (Fig. 2A). The results indicated that after four rounds of transfer, the levels of *CBF* transcripts obtained upon cold shock were significantly diminished. Moreover, when

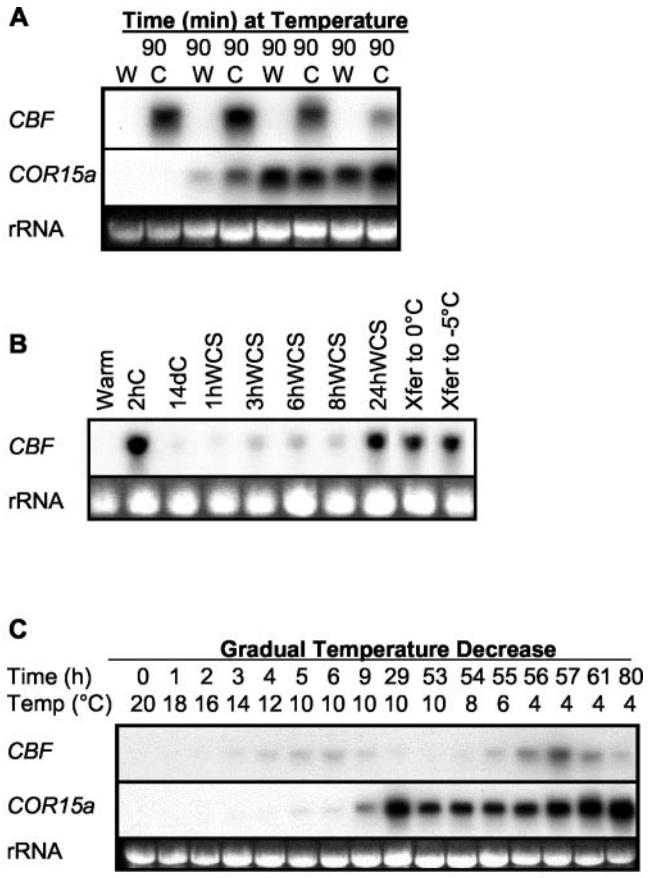

Figure 2. Desensitization and resensitization of the regulatory circuitry controlling accumulation of *CBF* transcripts. A, Plants were subjected to repeated transfer between 20°C (W) and 4°C (C), and *CBF* and *COR15a* transcript levels were determined by RNA-blot hybridization. B, *CBF* transcript levels of in plants that were cold acclimated at 4°C (C) for 14 d, returned to warm temperatures for the indicated times, and then subjected to a cold shock (WCS) at 4°C. *CBF* transcript levels were also determined in cold-acclimated plants (14 d) that were transferred (Xfer) to 0°C or −5°C 2 h. C, *CBF* and *COR15a* transcripts levels in plants cooled at 2°C h^{-1}. Plants were held at 10°C for 48 h before cooling to 4°C. The probe used hybridized with *CBF1*, *2*, and *3* transcripts. rRNA stained with ethidium bromide was used to compare loading in each experiment.

plants that had been cold-acclimated at 4°C for 14 d were returned to warm temperatures for 1 h and then abruptly transferred to 4°C, there was no detectable increase in *CBF* transcript levels (Fig. 2B). If, however, cold-acclimated plants were allowed to adjust to warm temperatures for 24 h and then transferred to 4°C, normal *CBF* transcript levels were attained. These data suggested that the cold-sensing mechanism became desensitized to 4°C upon extended incubation and that it could become resensitized to 4°C after 24 h at warm temperatures. The resensitization process took between 8 and 24 h, as cold-acclimated plants that had been returned to warm temperature for 3, 6, and 8 h were incapable of mounting a normal cold-shock induction of *CBF* transcripts (Fig. 2B).

Significantly, the desensitization that occurred upon exposure to 4°C did not eliminate the ability of the plants to sense and respond to further drops in temperature. When plants that had been cold-acclimated at 4°C for 14 d were directly transferred to 0°C or −5°C, an increase in *CBF* levels occurred (Fig. 2B). Also, desensitization was not unique to 4°C. When plants were subjected to a gradual decrease in temperature from 20°C to 10°C, accumulation of *CBF* transcripts occurred, but the levels declined upon continued exposure to this temperature (Fig. 2C). Upon renewed gradual decrease in temperature, *CBF* transcripts again increased. As in the cold-shock experiments, the levels of *CBF* transcripts attained at 4°C were greater than those attained at 10°C (Fig. 2C).

CBF Transcripts Have a Short Half-Life at Warm Temperatures

The results of the temperature transfer experiments described above indicated that that the half-life of the *CBF* transcripts at warm temperatures was very short, as no transcripts were detected 90 min after transferring plants from cold to warm temperatures (Fig. 2A). To estimate the half-life of the *CBF* transcripts, plants were transferred from cold to warm temperatures and the transcript levels were determined at 10-min intervals (Fig. 3). The results indicate that the *CBF* transcripts had a half-life of only 7.5 min at warm temperatures, a value that is among the shortest described for plant genes (Gutierrez et al., 2002). This value is a maximum estimate as it assumes that the promoters of the *CBF* genes become inactive within minutes of transferring plants from low to warm temperatures.

A 125-bp Region of the *CBF2* Promoter Contains Multiple *cis*-Acting Regulatory Elements Contributing to Cold-Responsive Gene Expression

Shinwari et al. (1998) reported that the promoters of *CBF1/DREB1b*, *CBF2/DREB1c*, and *CBF3/DREB1a*

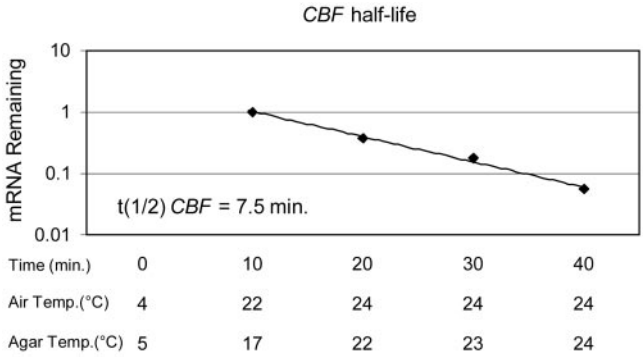

Figure 3. Estimation of *CBF* transcript half-life at warm temperatures. Plants that had been exposed to a low temperature (4°C) for 2 h were returned to a warm temperature (24°C) and *CBF* transcript levels were determined at 10-min intervals by RNA-blot hybridization. Air and agar temperatures in a plate were recorded during the experiment. Transcript levels for *eif-4A* did not change significantly during the time course and were used as a reference for loading. The probe for *CBF* hybridized with *CBF1*, *2*, and *3* transcripts.

are responsive to low temperatures. Our results confirmed this finding (Fig. 4). Approximately 1 kb of each *CBF* promoter (including the 5′-untranslated region up to the ATG start codon) was fused to the *β-glucuronidase* (*GUS*) reporter gene, transformed into Arabidopsis plants, and T_2 and T_3 populations of plants were analyzed for expression of the promoter::*GUS* fusions in response to low temperature. RNA-blot analysis indicated that the transcript levels for the reporter genes increased markedly in response to low temperatures (Fig. 4). However, surprisingly, histochemical staining of the plants for GUS activity did not reflect the differences in *GUS* transcript levels; staining was very similar in intensity regardless of whether the plants were grown at warm or low temperatures for varying lengths of time (up to 3 d; not shown). This suggested that the *CBF* promoters were active at normal warm temperatures during development of the seedlings. GUS activity resulting from *GUS* gene activation was detectable in the hypocotyl of seedlings 4 d after germination and could be detected throughout seedlings after 10 and 14 d of growth (not shown). Several different transgenic lines containing any of the three *CBF* promoters fused to *GUS* showed similar staining results. Whether the staining at later times is due to continued activation of the *CBF* promoters or GUS protein stability is uncertain. Regardless, GUS enzyme activity could not be used to faithfully reflect cold induction of the *CBF* promoters. Therefore, RNA-blot hybridizations were used for all further analysis of promoter activity.

A deletion analysis was conducted to identify sequences within the *CBF2* promoter that were involved in cold-regulated gene expression. We chose to work with *CBF2* based on the observation that transcripts for *CBF2* accumulated to higher levels than *CBF1* and *CBF3*, suggesting that the promoter might be more active and thus potentially easier to analyze than the others. One set of deletions removed sequences from the 5′ end of the promoter and included the 5′-untranslated region up to the ATG start codon (Fig. 5A). Another set of deletions removed sequences from the 3′ end beginning just upstream of the TATA sequence (Fig. 5B). With these later constructs, the *CBF2* promoter fragments were placed upstream of a minimal promoter fragment from the CaMV*35S* gene that provides the TATA sequence. In this vector, −46 *CaMV:GUS*, the minimal promoter drives expression of the *GUS* reporter gene. These constructs were designed to test whether any of the sequences that are conserved between the three *CBF/DREB1* promoters, designated boxes I to VI by Shinwari et al. (1998), were involved in cold responsiveness.

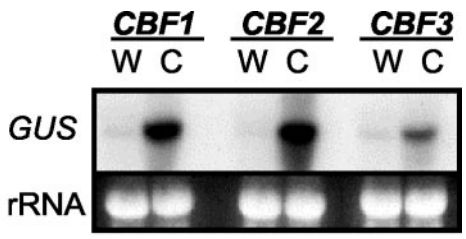

Figure 4. Activation of the *CBF1*, *2*, and *3* promoters in response to low temperatures. Representative transgenic lines of Arabidopsis carrying the *CBF1*, *2*, or *3* promoter fused to the *GUS* reporter gene were transferred from 22°C (W) to 4°C (C) for 2 h. The transcript levels of the gene fusions were determine using a probe for the *GUS* sequences.

The results indicated that 5′ deletions to −419, −369, −319, and −189 did not greatly impair the cold responsiveness of the *CBF2* promoter (Fig. 6A). Thus, boxes I, II, and III were not required for cold induction of the promoter (Fig. 5A). However, deletion of the promoter to −68, which removed boxes IV, V, and VI, resulted in almost complete elimination of cold responsiveness (Fig. 6A). Additional constructs indicated that 5′ deletions to −162, −109, and −94 also severely reduced responsiveness of the promoter (Fig. 6B). Thus, sequences between −189 and −162, which includes box IV (Fig. 5A), appeared to have an important role in cold-regulated expression of the promoter.

The sequence from −724 to −35 could impart cold-regulated gene expression when fused to the −46 *CaMV:GUS* reporter gene (Figs. 5B and 6C). Deletion from the 3′ end to −64 did not have a dramatic effect on the cold responsiveness, but deletion to −145 all but eliminated cold responsiveness of the promoter fragment (Fig. 6C). These results indicated that boxes I, II, III, and IV were insufficient to impart robust cold-regulated gene expression and that box V or VI or both might contain a cold-responsive element(s) (Fig. 5B).

The results of the 5′ and 3′ deletion analysis suggested that sequences between −189 and −65, which contained boxes IV, V, and VI, might be sufficient to impart cold-responsive gene expression (Fig., A and B). Dimers of the −189/−35 or −189/−65 sequences were responsive to low temperatures (Fig. 6D). Dimer fragments consisting of −189/−78 and −146/−64 sequences, which resulted in deletion of box IV and the right one-half of box VI, respectively (Fig. 5C), eliminated strong cold responsiveness (Fig. 6D). These results were consistent with the hypothesis that boxes IV and VI were involved in cold-regulated expression of the *CBF2* promoter (Fig. 5C). In addition, the possibility remained that box V was also required for robust cold responsiveness.

To confirm the importance of the boxes IV and VI sequences in cold-responsive gene expression, and to explore the importance of box V, mutations were introduced into these sequences in the context of the −189 5′ deletion construct and were tested for cold induction (Fig. 7). Whereas the wild-type −189 5′ deletion construct was strongly cold responsive (Fig. 6A), the mutant versions having the entire box IV (mboxIV) or right-hand portion of box VI (m4) sub-

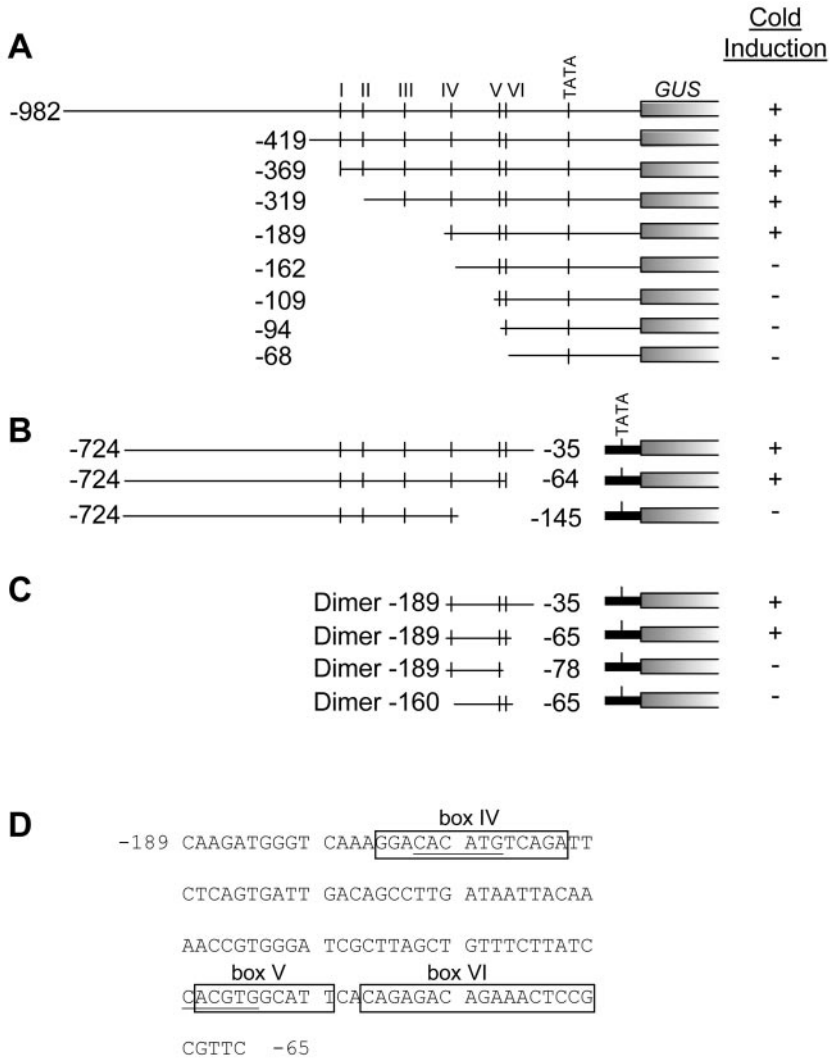

Figure 5. *CBF2* promoter fragments used to identify cold-regulatory elements. Each fragment was inserted into a binary vector upstream of the *GUS* reporter gene. Fragments that were deleted from the 3′ end were inserted into a binary vector upstream of the cauliflower mosaic virus (cauliflower mosaic virus) 35S minimal promoter (−46 minimal promoter) fused to *GUS*. A, Promoter fragments with deletions from the 5′ end. B, Promoter fragments with deletions from the 3′ end. C, Dimer constructs. D, Sequence of the 125-bp region of the *CBF2* promoter (positions −189 to −65) that imparts cold-regulated gene expression. The locations of the conserved boxes IV, V, and VI are indicated and the E-box/ABRE-like sequences are underlined. Relative levels of cold induction (data from Figs. 6–8) are indicated as "+" (strong) and "−" (weak).

stituted with alternative nucleotides displayed very low induction in response to low temperatures (Fig. 7). In contrast, mutagenesis of the left-hand portion of box VI (m3) or the E-box (CANNTG; Massari and Murre, 2000)/ABRE-like (CACGTG; Busk and Pages, 1998) sequences present at the left-side of box V (m2) had no obvious effects on cold-regulated gene expression.

In summary, the promoter analysis described above indicated that sequences within box IV and the right-hand side of box VI are involved in cold induction of the *CBF2* promoter. Therefore, these sequences were designated "Induction of CBF Expression region 1 and 2" (ICEr1 and ICEr2), respectively.

ICEr1 and ICEr2 are Involved in Responsiveness of the *CBF2* Promoter to Mechanical Agitation and Cycloheximide Treatment

Earlier work established that *CBF* transcripts accumulate rapidly in response to mechanical agitation (Gilmour et al., 1998) and the protein synthesis inhibitor cycloheximide (Qin, 2001). To determine whether this regulation involved the same sequences involved in cold induction of the *CBF2* promoter, we compared cold, mechanical, and cycloheximide induction of specific promoter constructs described above. The results indicated that as with cold, the ICEr1 and ICEr2 sequences were involved in mechanical and cycloheximide regulation of the promoter. Whereas the native promoter deleted 5′ to −189 and the 125-bp −189/−65 dimer fragment conferred responsiveness to treatments with cold, mechanical agitation, and cycloheximide, the site specific mutations m4 and mboxIV virtually eliminated responsiveness to these factors (Fig. 8).

DISCUSSION

The results reported here provide further insights into the regulation of *CBF* genes by low temperatures. One is that the cold-sensing mechanism that controls *CBF* expression does not require a cold shock to bring about the accumulation of *CBF* tran-

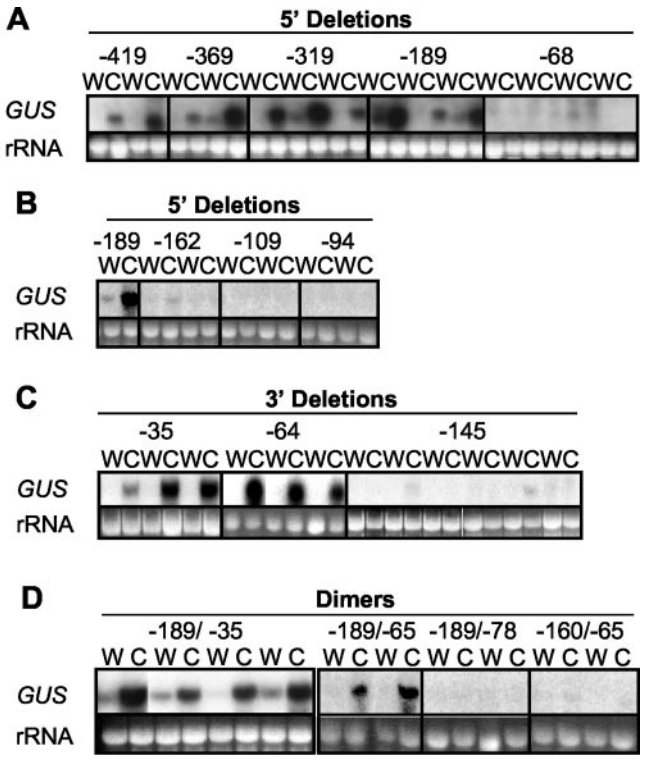

Figure 6. Activities of *CBF2::GUS* promoter constructs. RNA isolated from plants grown at 22°C (W) or grown at 22°C and transferred to 4°C for 2 h (C) was analyzed for *GUS* transcript levels by RNA-blot hybridization. A and B, Plants containing 5′ deletion constructs. C, Plants containing 3′ deletion constructs. D, Plants containing dimer constructs. rRNA stained with ethidium bromide was used to compare loading in each experiment.

scripts (Fig. 1). Instead, the thermosensing circuitry appears to monitor absolute temperature and act like a rheostat increasing output, i.e. the levels of *CBF* transcripts, with greater degrees of input, i.e. lower temperatures. However, the output from the circuit (which could involve changes in rates of *CBF* transcription and rates of *CBF* transcript turnover) does not remain constant with time at a given temperature. Within a few hours of exposure to a temperature, the thermosensing circuitry becomes desensitized to that temperature and produces less output—lower levels of *CBF* transcripts (Fig. 1). Significantly, this apparent desensitization does not preclude a robust response to a further decrease in temperature. Plants that had been adapted to 4°C for 14 d and had low levels of *CBF* transcripts produced high levels of *CBF* transcripts when transferred to 0°C or −5°C (Fig. 2). In contrast, when plants that had been adapted to 4°C for 14 d were returned to warm temperatures for 1 to 8 h, and then abruptly returned to 4°C, *CBF* transcripts showed little increase in accumulation; resensitizing 4°C-adapted plants to 4°C took between 8 and 24 h of exposure to 22°C (Fig. 2). These results indicate that the thermosensing circuitry controlling *CBF* expression involves a low temperature "memory" component affecting desensitization.

At present, we know nothing of the nature of the putative thermosensing memory component. However, a "cold memory" has been described for cold-induced calcium flux in Arabidopsis (Knight et al., 1996). Transfer of Arabidopsis plants to low temperatures results in a rapid, transient increase in free calcium in the cytoplasm, which comes from extracellular and intracellular calcium stores (Knight et al., 1996; Knight and Knight, 2000). Knight et al. (1996) have shown that the calcium signature—the peak levels and duration of the calcium influx—is altered upon cold acclimation, becoming diminished in magnitude and prolonged in length. The significance here is that the expression of certain cold-regulated genes, including *COR* and other *CBF*-targeted genes, appears to involve the action of calcium as second messenger. When cold-induced calcium influx was inhibited using chemicals or pharmacological agents, expression of the *COR* genes was impaired; when chemical and pharmacological agents were used to raise intracellular calcium levels at warm temperatures, *COR* gene expression was induced (Knight et al., 1996; Tahtiharju et al., 1997). Thus, an interesting possibility raised is that the desensitization "memory" that we describe here may be related to the calcium cold memory described by Knight et al. (1996).

What are the molecular components of the thermosensing circuitry that regulate accumulation of *CBF* transcripts? At present, our understanding in this area is limited, but proteins that are potential components of the thermosensing mechanism have been identified by Zhu and colleagues in screens for mutants altered in low-temperature expression of *RD29A*, a CBF-targeted gene (Ishitani et al., 1997). HOS1 (Lee et al., 2001) and FRY2 (Xiong et al., 2002), a novel RING finger protein and novel transcriptional repressor, respectively, appear to be negative regulators of *CBF* expression whereas LOS4, a DEAD-box RNA helicase, appears to be a positive regulator (Gong et al., 2002). In addition, mutant analysis indicates that the thermosensing circuitry may include a negative feedback loop that includes the *CBF* proteins themselves or their downstream targets (Guo et al., 2002).

Key components of the regulatory mechanism that control *CBF* expression include the transcription factors that bind to the promoter and control transcription. One such factor, ICE1, was recently reported (Chinnusamy et al., 2003). ICE1 encodes a MYC-like bHLH protein that appears to have a fundamental role in the regulation of *CBF3*. A dominant-negative allele of *ICE1*, *ice1*, results in almost complete elimination of *CBF3* transcript accumulation in response to low temperatures. Significantly, however, accumulation of *CBF2* transcripts in response to low temperatures is little affected by the *ice1* mutation (and may, in fact, be somewhat greater in the *ice1* mutant).

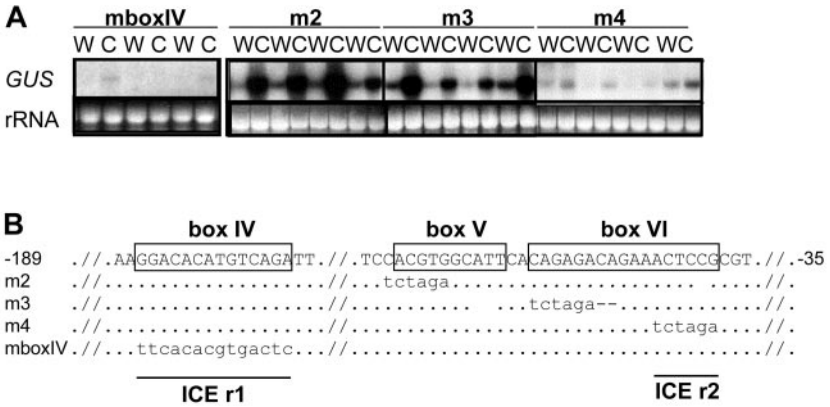

Figure 7. Mutational analysis identifying regions of the *CBF2* promoter involved in cold-regulated gene expression. A, *CBF2::GUS* promoter constructs with mutations in boxes IV (mboxIV), V (m2), and VI (m3 and m4) were tested for cold responsiveness by transferring plants from 22°C (W) to 4°C (C) for 2 h and determining *GUS* transcript levels by RNA-blot hybridization. rRNA stained with ethidium bromide was used to compare loading. B, Sequence corresponding to wild-type and mutated (m2, m3, m4, and mboxIV) *CBF2::GUS* promoter constructs. Regions identified as required for cold induction are underlined and designated ICEr1 and ICEr2.

Thus, ICE1 would not appear to have a critical role in *CBF2* expression at low temperatures.

As a step toward identifying transcription factors involved in *CBF2* expression, we identified a 125-bp promoter segment that is sufficient to impart cold-responsive gene expression (Figs. 5–7). Within this 125-bp segment are two regions, designated ICEr1 and ICEr2, that by themselves are only weakly responsive to low temperatures, but in combination, impart a robust cold response. There are no obvious known transcription factor-binding sites within the ICEr2 sequence, but ICEr1 contains the sequence CACATG, which includes a consensus recognition site for bHLH proteins, CANNTG (Massari and Murre, 2000). Therefore, ICEr1 is a potential binding site for the ICE1 protein. However, as alluded to above, the *ice1* mutation has little effect on *CBF2* transcript accumulation at low temperatures. Thus, it is unclear whether ICE1 binds to ICEr1 in vivo and contributes to *CBF2* expression. However, Arabidopsis encodes an estimated 139 bHLH proteins (Riechmann and Ratcliffe, 2000), some of which have DNA-binding domains that are similar to that present in ICE1 (Chinnusamy et al., 2003). These must be considered candidates for ICEr1-binding proteins. Another potential bHLH protein-binding site, CACGTG, which is also a potential abscisic acid-responsive element and bZIP protein-binding site (Busk and Pages, 1998), overlaps the left-hand end of box V. However, mutations in this sequence, in the context of the −189 5′ deletion, had no apparent effect on cold responsiveness and thus, a role for this sequence in *CBF2* expression is not evident.

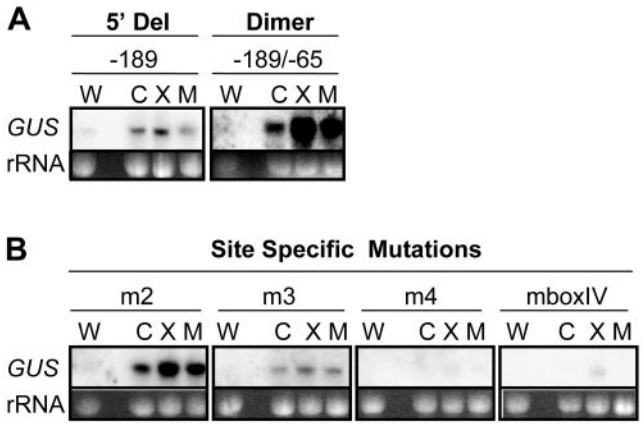

Figure 8. Sequences of the *CBF2* promoter involved in cold, cycloheximide, and mechanical responsiveness. Plants containing *CBF2* promoter constructs were grown at 22°C (W) and then exposed to 4°C (C) for 2 h or treated with cycloheximide (X) or mechanical agitation (M) as described in "Materials and Methods." RNA was isolated and *GUS* transcript levels were determined by RNA-blot hybridization. A, Plants containing the 5′ deletion to −189 (5′Del) or the −189/−65 dimer (Dimer) construct. B, Plants containing the m2, m3, m4, and mboxIV mutations. rRNA stained with ethidium bromide was used to compare loading in each experiment.

The ICEr1 and ICEr2 sequences are not only involved in cold responsiveness of the *CBF2* promoter, but also in induction of the promoter by mechanical agitation and cycloheximide. Whether cold, mechanical agitation, or cycloheximide mediate their action through the same regulatory system remains to be determined. However, it is of interest that mechanical agitation, like low temperatures, causes a transient increase in cytoplasmic calcium levels (Knight et al., 1992). Thus, a plausible hypothesis is that cold and mechanical stress cause a calcium flux that stimulates *CBF* transcription. Whether the increases in calcium brought on by cold and mechanical stress are mediated through the same mechanism is unknown. However, in this regard, it is noteworthy that the activity of an onion-mechanosensitive calcium-selective channel has been shown to increase as tem-

perature is lowered to about 6°C (Ding and Pickard, 1993).

A relationship between calcium flux and cycloheximide-induced gene expression also exists. Berberich and Kusano (1997) have found that transcripts for m*lip15*, a cold-inducible maize (*Zea mays*) gene encoding a putative bZIP transcription factor, accumulate in response to cycloheximide and that chelators of calcium reduce m*lip15* transcript accumulation in response to cold and cycloheximide treatments. Thus, cycloheximide, like mechanical agitation, may induce *CBF2* expression by causing an increase in intracellular calcium levels. The fact that cycloheximide inhibits translation raises the possibility that the mechanism controlling cold-induced calcium flux might involve action of a short-lived repressor protein that keeps a calcium channel "closed" in the absence of a low temperature or mechanical stimulus.

A final point regards a possible relationship between the regulation of *CBF* genes and the Arabidopsis *TCH4* gene, which encodes a xyloglucan endotransglycosylase (Purugganan et al., 1997). Like the *CBF* genes, *TCH4* is induced in response to cold temperature and touch (mechanical agitation; Polisensky and Braam, 1996). Iliev et al. (2002) defined a 102-bp region of the *TCH4* promoter that is sufficient to impart cold and touch responsiveness. Within this region is a 24-bp segment of the *TCH4* promoter (positions −138 to −114) that is 66% identical in sequence to the *CBF2* promoter between positions −189 and −165. Significantly, this region of the *CBF2* promoter contains the ICEr1 sequence that is involved in cold and mechanical responsiveness. These observations suggest that *TCH4* may be regulated, at least in part, by the same cold- and mechanical-regulatory system that controls *CBF2* gene expression.

MATERIALS AND METHODS

Plant Material, Growth, and Treatment Conditions

Arabidopsis ecotype Wassilewskija-2 and transgenic plants in the Wassilewskija-2 background were grown in controlled environment chambers at 22°C under constant illumination from cool-white fluorescent lights (100–125 μmol m^{-2} s^{-1}) in Baccto planting mix (Michigan Peat, Houston). Pots were subirrigated with deionized water as needed. Plants were also grown on solid agar medium that contained Gamborg's B5 nutrients (Invitrogen, Carlsbad, CA) and 1% (w/v) phytoagar (Invitrogen) for various treatments. Experiments were performed on seedlings that were 10- to 12-d-old. Cold-shock treatments involved transfer of plants from a 22°C chamber to a 4°C chamber with dim light (approximately 25 μmol m^{-2} s^{-1}). Gradual temperature decreases were achieved by decreasing the temperature of the growth chamber 0.5°C every 15 min until a desired temperature was reached. Treatments with cycloheximide were performed by growing seedlings on filter papers that had been placed on top of the agar so that the seedlings could be lifted off the plate with minimal damage or mechanical stress. The seedlings were then floated on a solution of cycloheximide (10 μg mL^{-1}) in covered dishes for 2 h. Mechanical treatment involved tapping plates on a bench top for 15 min before harvesting tissue.

Constructs and Plant Transformation

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

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

The DNAs were transferred to Arabidopsis via a whole-plant dipping method similar to that described by Clough and Bent (1998). Plants containing transferred DNA were identified by germinating seeds on medium containing kanamycin (50 mg L^{-1}). These kanamycin-resistant seedlings were grown to maturity and the next generation seeds (T_2) or T_3 seeds were used for experiments.

Staining for GUS Activity

To stain plant tissue to determine location of GUS activity, experimentally treated plants were immersed in a GUS-staining solution as described by Jefferson et al. (1987) and were incubated overnight at 37°C. Tissue was cleared with several rinses of 70% (v/v) ethanol to aid in the localization of the staining.

RNA Isolation and Hybridization

Total RNA was extracted from plant material with the use of RNeasy Plant Mini kits (Qiagen, Valencia, CA) with modifications. To obtain adequate and consistent yields with the kit, the amount of starting plant tissue was doubled. Subsequently, the amount of extraction buffer (RLT) was also doubled. The remaining procedure was performed as described in the Qiagen manual.

Northern transfers were prepared and hybridized as described (Hajela et al., 1990) using normal high-stringency wash conditions (Stockinger et al., 1997).

Probes for specific *CBF* genes and *GUS* were prepared as previously described by Baker et al. (1994) and Gilmour et al. (1998). Probes were labeled with ^{32}P by random priming (Feinberg and Vogelstein, 1983).

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LITERATURE CITED

Artus NN, Uemura M, Steponkus PL, Gilmour SJ, Lin C, Thomashow MF (1996) Constitutive expression of the cold-regulated *Arabidopsis thaliana* COR15a gene affects both chloroplast and protoplast freezing tolerance. Proc Natl Acad Sci USA **93:** 13404–13409

Baker SS, Wilhelm KS, Thomashow MF (1994) The 5′ region of *Arabidopsis thaliana* cor15a has cis-acting elements that confer cold-, drought- and ABA-regulated gene expression. Plant Mol Biol **24:** 701–713

Berberich T, Kusano T (1997) Cycloheximide induces a subset of low temperature-inducible genes in maize. Mol Gen Genet **254:** 275–283

Busk PK, Pages M (1998) Regulation of abscisic acid-induced transcription. Plant Mol Biol **37:** 425–435

Chinnusamy V, Ohta M, Kanrar S, Lee BH, Hong X, Agarwal M, Zhu JK (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in Arabidopsis. Genes Dev **17:** 1043–1054

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J **16:** 735–743

Ding JP, Pickard BG (1993) Modulation of mechanosensitive calcium-selective cation channels by temperature. Plant J **3:** 713–720

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem **132:** 6–13

Gilmour SJ, Sebolt AM, Salazar MP, Everard JD, Thomashow MF (2000) Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiol **124:** 1854–1865

Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM, Thomashow MF (1998) Low temperature regulation of the Arabidopsis CBF family of AP2 transcriptional activators as an early step in cold-induced COR gene expression. Plant J **16:** 433–442

Gong Z, Lee H, Xiong L, Jagendorf A, Stevenson B, Zhu JK (2002) RNA helicase-like protein as an early regulator of transcription factors for plant chilling and freezing tolerance. Proc Natl Acad Sci USA **99:** 11507–11512

Guo Y, Xiong L, Ishitani M, Zhu JK (2002) An Arabidopsis mutation in translation elongation factor 2 causes superinduction of CBF/DREB1 transcription factor genes but blocks the induction of their downstream targets under low temperatures. Proc Natl Acad Sci USA **99:** 7786–7791

Gutierrez RA, Ewing RM, Cherry JM, Green PJ (2002) Identification of unstable transcripts in Arabidopsis by cDNA microarray analysis: rapid decay is associated with a group of touch- and specific clock-controlled genes. Proc Natl Acad Sci USA **99:** 11513–11518

Guy CL (1990) Cold acclimation and freezing stress tolerance: role of protein metabolism. Annu Rev Plant Physiol Plant Mol Biol **41:** 187–223

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

Iliev EA, Xu W, Polisensky DH, Oh MH, Torisky RS, Clouse SD, Braam J (2002) Transcriptional and posttranscriptional regulation of Arabidopsis TCH4 expression by diverse stimuli: roles of cis regions and brassinosteroids. Plant Physiol **130:** 770–783

Ishitani M, Xiong L, Stevenson B, Zhu JK (1997) Genetic analysis of osmotic and cold stress signal transduction in Arabidopsis: interactions and convergence of abscisic acid-dependent and abscisic acid-independent pathways. Plant Cell **9:** 1935–1949

Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF (1998) Arabidopsis CBF1 overexpression induces COR genes and enhances freezing tolerance. Science **280:** 104–106

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J **6:** 3901–3907

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nat Biotechnol **17:** 287–291

Knight H, Knight MR (2000) Imaging spatial and cellular characteristics of low temperature calcium signature after cold acclimation in Arabidopsis. J Exp Bot **51:** 1679–1686

Knight H, Trewavas AJ, Knight MR (1996) Cold calcium signaling in Arabidopsis involves two cellular pools and a change in calcium signature after acclimation. Plant Cell **8:** 489–503

Knight MR, Smith SM, Trewavas AJ (1992) Wind-induced plant motion immediately increases cytosolic calcium. Proc Natl Acad Sci USA **89:** 4967–4971

Lee HJ, Xiong LM, Gong ZZ, Ishitani M, Stevenson B, Zhu JK (2001) The Arabidopsis HOS1 gene negatively regulates cold signal transduction and encodes a RING finger protein that displays cold-regulated nucleocytoplasmic partitioning. Genes Dev **15:** 912–924

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low temperature-responsive gene expression, respectively, in Arabidopsis. Plant Cell **10:** 1391–1406

Massari ME, Murre C (2000) Helix-loop-helix proteins: regulators of transcription in eukaryotic organisms. Mol Cell Biol **20:** 429–440

Medina J, Bargues M, Terol J, Perez-Alonso M, Salinas J (1999) The Arabidopsis CBF gene family is composed of three genes encoding AP2 domain-containing proteins whose expression is regulated by low temperature but not by abscisic acid or dehydration. Plant Physiol **119:** 463–470

Nanjo T, Kobayashi M, Yoshiba Y, Kakubari Y, Yamaguchi-Shinozaki K, Shinozaki K (1999) Antisense suppression of proline degradation improves tolerance to freezing and salinity in *Arabidopsis thaliana*. FEBS Lett **461:** 205–210

Polisensky DH, Braam J (1996) Cold-shock regulation of the Arabidopsis TCH genes and the effects of modulating intracellular calcium levels. Plant Physiol **111:** 1271–1279

Purugganan MM, Braam J, Fry SC (1997) The Arabidopsis TCH4 xyloglucan endotransglycosylase: substrate specificity, pH optimum, and cold tolerance. Plant Physiol **115:** 181–190

Qin H (2001) Genetic analysis of a CBF-mediated signaling pathway in *Arabidopsis thaliana*. MS thesis. Michigan State University, East Lansing

Riechmann JL, Meyerowitz EM (1998) The AP2/EREBP family of plant transcription factors. Biol Chem **379:** 633–646

Riechmann JL, Ratcliffe OJ (2000) A genomic perspective on plant transcription factors. Curr Opin Plant Biol **3:** 423–434

Sambrook J, Fritsch E, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Shinwari ZK, Nakashima K, Miura S, Kasuga M, Seki M, Yamaguchi-Shinozaki K, Shinozaki K (1998) An Arabidopsis gene family encoding DRE/CRT binding proteins involved in low-temperature-responsive gene expression. Biochem Biophys Res Commun **250:** 161–170

Steponkus PL, Uemura M, Joseph RA, Gilmour SJ, Thomashow MF (1998) Mode of action of the COR15a gene on the freezing tolerance of *Arabidopsis thaliana*. Proc Natl Acad Sci USA **95:** 14570–14575

Stockinger EJ, Gilmour SJ, Thomashow MF (1997) *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proc Natl Acad Sci USA **94:** 1035–1040

Tahtiharju S, Sangwan V, Monroy AF, Dhindsa RS, Borg M (1997) The induction of kin genes in cold-acclimating *Arabidopsis thaliana*: evidence of a role for calcium. Planta **203:** 442–447

Taji T, Ohsumi C, Iuchi S, Seki M, Kasuga M, Kobayashi M, Yamaguchi-Shinozaki K, Shinozaki K (2002) Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. Plant J **29:** 417–426

Thomashow MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu Rev Plant Physiol Plant Mol Biol **50:** 571–599

Thomashow MF (2001) So what's new in the field of plant cold acclimation? Lots! Plant Physiol **125:** 89–93

Xiong L, Lee H, Ishitani M, Tanaka Y, Stevenson B, Koiwa H, Bressan RA, Hasegawa PM, Zhu JK (2002) Repression of stress-responsive genes by FIERY2, a novel transcriptional regulator in Arabidopsis. Proc Natl Acad Sci USA **99:** 10899–10904

Yamaguchi-Shinozaki K, Shinozaki K (1994) A novel cis-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell **6:** 251–264