Low Temperature Induction of Arabidopsis *CBF1*, *2*, and *3* Is Gated by the Circadian Clock[1]

Sarah G. Fowler[2], Daniel Cook[2,3], and Michael F. Thomashow*

Michigan State University–U.S. Department of Energy Plant Research Laboratory (S.G.F., D.C., M.F.T.), and Department of Crop and Soil Sciences (M.F.T.), Michigan State University, East Lansing, Michigan 48824

Exposing Arabidopsis (*Arabidopsis thaliana*) plants to low temperature results in rapid induction of *CBF1*, *2*, and *3* (*CBF1-3*; also known as *DREB1B*, *C*, and *A*, respectively), which encode transcriptional activators that induce expression of a battery of genes that increase plant freezing and chilling tolerance. Recently, it has been shown that basal levels of *CBF3* transcripts and those of certain CBF-regulated genes exhibit circadian cycling. Here, we further explored the regulation of *CBF1-3* by the circadian clock. The results indicated that the extent to which *CBF1-3* transcripts accumulated in response to low temperature was dependent on the time of day that the plants were exposed to low temperature and that this was regulated by the circadian clock. The highest and lowest levels of cold-induced *CBF1-3* transcript accumulation occurred at 4 and 16 h after subjective dawn, respectively. An analysis of *CBF2* promoter-reporter gene fusions indicated that this control included transcriptional regulation. In addition, the cold responsiveness of *RAV1* and *ZAT12*, genes that are cold induced in parallel with *CBF1-3*, was also subject to circadian regulation. However, whereas the maximum level of cold-induced *RAV1* transcript accumulation occurred at the same time of day as did *CBF1-3* transcripts, that of *ZAT12* was in reverse phase, i.e. the highest level of cold-induced *ZAT12* transcript accumulation occurred 16 h after subjective dawn. These results indicate that cold-induced expression of *CBF1-3*, *RAV1*, and *ZAT12* is gated by the circadian clock and suggest that this regulation likely occurs through at least two nonidentical (though potentially overlapping) signaling pathways.

Many plants have the ability to sense low temperature and respond by activating mechanisms that lead to an increase in freezing tolerance, an adaptive response known as cold acclimation (Thomashow, 1999; Smallwood and Bowles, 2002). At present, the best understood genetic system that has a role in cold acclimation is the Arabidopsis (*Arabidopsis thaliana*) CBF cold-response pathway (Thomashow, 2001). Exposing Arabidopsis plants to low temperature results in rapid induction of a small family of transcriptional activators known either as *CBF1*, *2*, and *3* (*CBF1-3*; Stockinger et al., 1997; Gilmour et al., 1998; Medina et al., 1999) or as *DREB1B*, *C*, and *A*, respectively (Liu et al., 1998). These transcription factors, which belong to the AP2/ERF domain family of DNA-binding proteins (Riechmann and Meyerowitz, 1998), recognize a cis-acting regulatory element known as the C-repeat/dehydration response element (CRT/DRE; Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger et al., 1997) that is present in the promoters of many cold-inducible genes such as *COR15A* and *COR78* (also known as *RD29A* and *LTI78*). Transgenic plants overexpressing *CBF1*, *2*, or *3* constitutively express CBF-targeted cold-induced genes, the CBF regulon, and exhibit an increase in freezing tolerance that is independent of a cold stimulus (Jaglo-Ottosen et al., 1998; Liu et al., 1998).

Transcripts for *CBF1-3* accumulate not only in response to low temperature but also in response to mechanical agitation (Gilmour et al., 1998), abscisic acid (Knight et al., 2004), and the inhibition of protein synthesis (Zarka et al., 2003). In addition, *CBF3* is regulated by the circadian clock; *CBF3* transcript levels display circadian-regulated cycling at warm temperatures, reaching a peak at Zeitgeber time ZT4 and a minimum at ZT16 (Harmer et al., 2000), 4 and 16 h after subjective dawn, respectively. The transcript levels for two CBF-targeted genes, *COR15B* and *COR6.6*, also cycle at warm temperature but with a peak phase delayed by approximately 8 h from that of *CBF3*, a situation that presumably reflects the time required for *CBF3* transcripts to be translated and produce peak levels of CBF3 protein.

The cold-regulated *CCR1* and *CCR2* genes of Arabidopsis are also regulated by the circadian clock. Peak clock-regulated expression of these genes occurs 11 h after dawn (Carpenter et al., 1994). Low temperature causes a small increase in transcripts levels for *CCR1* and *CCR2* at both the peak and trough of the circadian cycle (Carpenter et al., 1994). However, long-term

[1] This work was supported by grants from the National Science Foundation Plant Genome project (grant no. DBI 0110124), the U.S. Department of Energy (grant no. DEFG0291ER20021), and the Michigan Agricultural Experiment Station.

[2] These authors contributed equally to the paper.

[3] Present address: U.S. Department of Agriculture Agricultural Research Service, Natural Products Utilization Research Unit, University, MS 38677.

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

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.104.058354.

continuous cold treatments interrupt cycling of clock-regulated expression of *CCR1* and *CCR2* (Kreps and Simon, 1997). Furthermore, a cold pulse during free-running conditions delays the phase of cycling of *CCR1* and *CCR2* transcript levels.

Circadian rhythms in chilling and freezing tolerance have also been described for several plant species, including cotton (*Gossypium hirsutum*; Rikin et al., 1993) and soybean (*Glycine max*; Couderchet and Koukkari, 1987). In both these species, the clock regulates development of a low temperature-resistant phase that peaks at the end of the light phase. Although cycling of low-temperature tolerance has not been observed in Arabidopsis, it is of interest to note that the phase of low-temperature resistance in soybean (Couderchet and Koukkari, 1987) and cotton (Rikin et al., 1993) coincides with the clock-regulated peak of CBF-target gene induction observed in Arabidopsis (Harmer et al., 2000).

Circadian clocks show self-sustained oscillations under constant conditions but can be entrained to match local time by external environmental cues (Devlin and Kay, 2001). Light is the major cue entraining the circadian clock to environmental cycles in plants, but temperature may also act as an entraining stimulus (Bünning, 1973). Rhythmic temperature changes have been shown to induce cycling of clock-regulated genes in Arabidopsis (Somers et al., 1998; Michael et al., 2003) and *Sinapis alba* plants (Heintzen et al., 1994) maintained in continuous light (LL), and in pea (*Pisum sativum*; Kloppstech et al., 1991) and barley (*Hordeum vulgare*; Beator and Kloppstech, 1992) plants maintained in continuous dark. Significantly, the cycling of some clock-regulated genes shows differing sensitivities to light and temperature entrainment, suggesting the existence of two molecular oscillators that can be distinguished based on sensitivity to temperature (Michael et al., 2003).

Here, we further explore interactions between the circadian clock and the CBF cold-response pathway. The results indicate that cold-induced expression of *CBF1-3* as well as *RAV1* and *ZAT12*, two cold-responsive genes that are induced in parallel with *CBF1-3*, is gated by the circadian clock and that this regulation is likely to involve at least two nonidentical (though potentially overlapping) signaling pathways, which in the case of *CBF2* involve transcriptional regulation.

RESULTS

The Circadian Clock Gates Low Temperature-Induced Accumulation of *CBF1-3* Transcripts in Response to Low Temperature

Harmer et al. (2000) showed that transcript levels for *CBF3* exhibit circadian-regulated cycling at warm temperatures. This finding raised the question of whether the circadian phase at which plants were transferred to low temperature would affect the degree to which *CBF1-3* transcripts accumulated upon exposing plants to low temperature. To begin to test this, Arabidopsis plants that had been grown at 24°C for 14 d on a 12-h photoperiod (12 h light/12 h dark) were transferred to low temperature for 1, 4, 8, and 24 h at either ZT4 or ZT16 (4 or 16 h, respectively, after dawn) and the levels of *CBF1-3* transcripts determined. These time periods were chosen, as Harmer et al. (2000) found them to correspond to the peak and trough of *CBF3* expression in plants grown at constant warm temperature. The results indicated that the level of cold-induced accumulation of the *CBF1-3* transcripts did depend on the time of day at which the transfer was made (Fig. 1). Transferring plants at ZT4 resulted in much greater accumulation of transcripts than transfer at ZT16. The results also indicated that when plants were kept at warm temperature, the levels of *CBF1-3* transcripts remained very low. No cycling of *CBF3* was detected in the warm samples, presumably due to the low level sensitivity of the northern analysis.

The results of this experiment were consistent with the circadian clock gating the cold responsiveness of *CBF1-3* expression. However, the differences in cold-induced accumulation of *CBF1-3* transcripts could also have been due to the presence and absence of light at the two harvesting times, rather than the influence of the circadian clock. To rule out this possibility, Arabidopsis plants that had been grown under a 12-h photoperiod at 24°C for 14 d were transferred to LL at ZT0 and then exposed to low temperature (4°C, LL) for 1, 4, 8, and 24 h, at 6-h intervals beginning at ZT4. The level of *CBF1-3* total transcripts was then determined using a probe prepared against the entire *CBF2* coding sequence, which hybridizes with *CBF1-3*. Again, the results (Fig. 2A) clearly showed a cycling in the degree to which *CBF1-3* transcripts accumulated in response to low temperature. As in the previous experiments, there was a peak of responsiveness at ZT4 and a trough at ZT16. This cycling continued with an approximately 24-h period for the next 36 h (the period of the cycling appeared to shorten during the second subjective

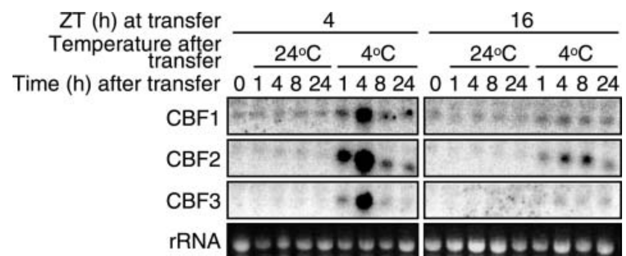

Figure 1. The extent to which *CBF1-3* transcripts accumulate in response to low temperature depends on the time of day at which plants are exposed to the cold. Arabidopsis plants were grown in a 12:12 photoperiod at 24°C. Plates were transferred to low temperature (4°C) at either ZT4 or ZT16 and samples harvested after the indicated times along with samples from plates that had been maintained at 24°C. RNA gel blots were prepared from total RNA and hybridized with gene-specific probes for *CBF1-3*. rRNA stained with ethidium bromide was used to compare loading.

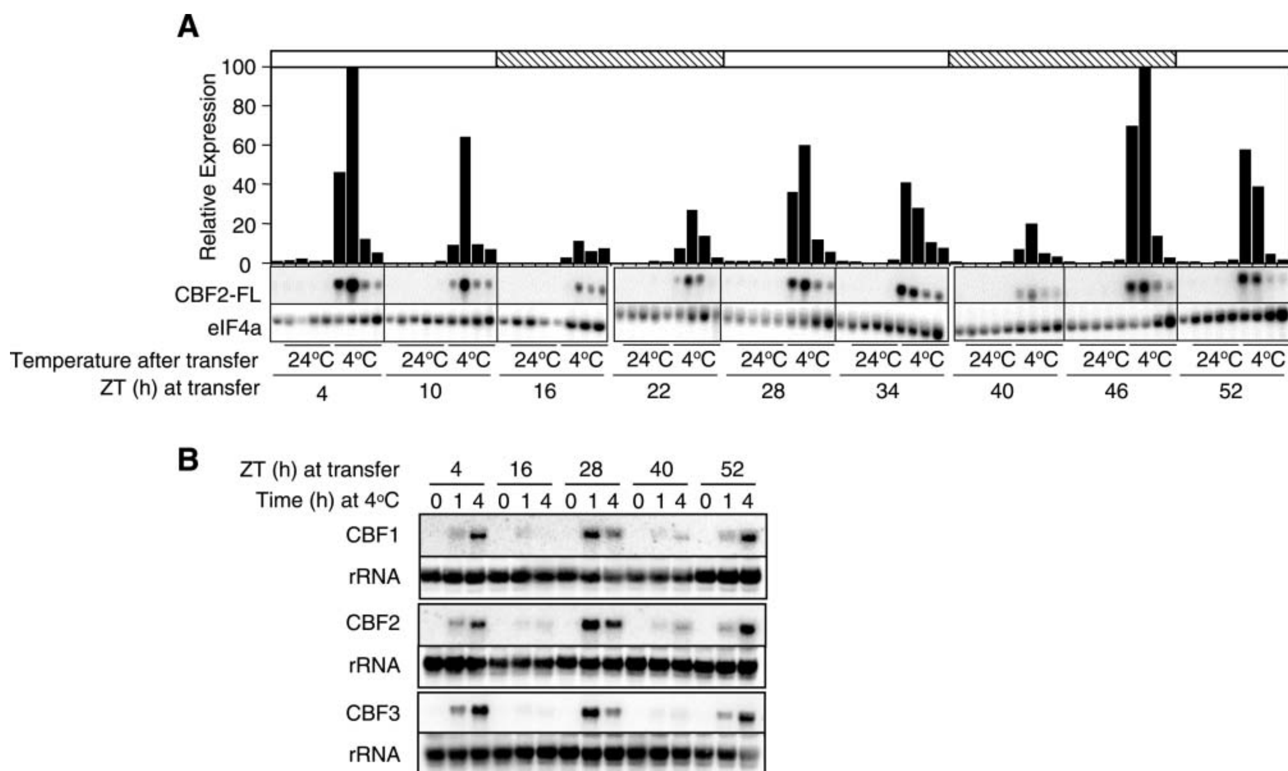

Figure 2. The circadian clock gates *CBF1-3* expression levels in response to low temperature. Arabidopsis plants were grown in a 12:12 photoperiod at 24°C and then released into LL at ZT0. Plants were then transferred to low temperature (4°C) at 6-h intervals beginning at ZT4. Samples were harvested from the cold-treated plants (4°C) and also from plants held at 24°C after 1, 4, 8, and 24 h (shown left to right above each temperature label). A, RNA gel blots were prepared from total RNA and hybridized with a full-length *CBF2* probe (CBF2-FL) that cross-hybridizes with *CBF1* and *CBF3* transcripts. Results are presented as a proportion of the highest value after normalization with respect to *eIF4a* expression levels. White and hatched boxes indicate subjective day and night, respectively. The first lane for each ZT time sample represents samples harvested before temperature treatment. B, RNA gel blots prepared from selected RNA samples derived from a repeat of experiment (A) above were hybridized with gene-specific probes for *CBF1-3*.

night). In a repeat of this experiment (Fig. 2B), the transcript levels for the individual *CBF1-3* transcripts were determined. Again, there was a peak and trough of responsiveness at ZT4 and ZT16, respectively, which continued to cycle for at least 52 h.

One additional experiment to confirm that the cold responsiveness of *CBF1-3* was gated by the circadian clock was to examine cold-induced accumulation of *CBF1-3* transcripts in plants in which circadian cycling had been abolished. In particular, the protein CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), a Myb-related transcriptional activator (Wang et al., 1997), is thought to be a component of the central circadian oscillator (Wang and Tobin, 1998; Green and Tobin, 2002; Mizoguchi et al., 2002). Transgenic plants constitutively expressing *CCA1* (*CCA1*-OX) exhibit arrhythmicity of all tested circadian rhythms (Schaffer et al., 1998; Wang and Tobin, 1998; Eriksson and Millar, 2003). Thus, we examined expression of *CBF1-3* in *CCA1*-OX transgenic plants. Plants were entrained in a 12-h photoperiod, released into LL at ZT0, and then transferred to 4°C for 1, 4, and 24 h at 12-h intervals, beginning at ZT4. Total *CBF1-3* transcript levels were then determined using the *CBF2*-FL probe. The results indicated that there was no cycling of *CBF1-3* transcript accumulation in response to low temperature (Fig. 3). Abolition of the cycling of cold-induced accumulation of *CBF1-3* transcripts in the *CCA1*-OX plants confirmed that a functional circadian clock is required to gate the response of *CBF1-3* expression to low temperature.

Circadian Gating of *CBF1-3* Cold Responsiveness Involves Transcriptional Regulation

The circadian gating of *CBF1-3* cold responsiveness could involve transcriptional control, posttranscriptional control, or both. To test whether the gating involved transcriptional regulation, we examined transgenic Arabidopsis lines that expressed the β-glucuronidase (*GUS*) reporter gene under the control of a 1-kb fragment of the *CBF2* promoter (CBF2::GUS). This gene fusion had previously been shown to be cold regulated (Zarka et al., 2003). After

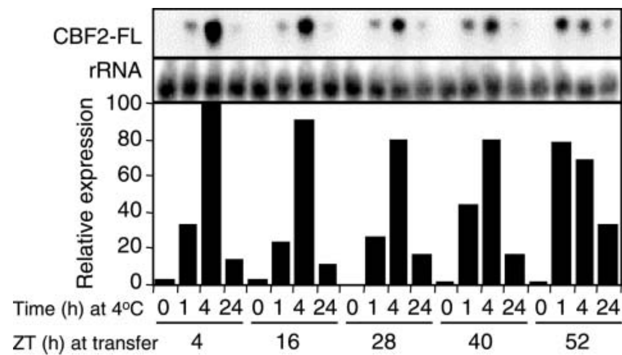

Figure 3. Constitutive expression of *CCA1* abolishes gating by the circadian clock of *CBF1-3* induction in response to low temperature. *CCA1*-OX plants were grown under a 12-h photoperiod at 24°C, transferred to LL at ZT0, and then exposed to low temperature (4°C, LL) for the indicated periods at 12-h intervals beginning at ZT4. These samples were grown and harvested concurrently with the wild-type samples shown in Figure 1. Total RNA was isolated and RNA blots prepared, which were hybridized with the CBF2-FL probe. In the bottom portion of the figure, the hybridization results are presented as a proportion of the highest value after normalization with respect to 25S rRNA expression levels.

entrainment in a 12-h photoperiod and release into LL at ZT0, CBF2::GUS plants were transferred to 4°C at ZT4 and 12-h intervals thereafter, and expression of the CBF2::GUS gene fusion was determined after 0, 1, 4, 8, and 24 h of cold treatment. The results (Fig. 4, A and B) indicated that GUS transcripts accumulated in response to low temperature and that the level of accumulation exhibited cycling in parallel with the *CBF2* transcripts, although the amplitude of cycling observed for the CBF2::GUS transcripts was much less than that observed for the endogenous *CBF1-3* transcripts. Two additional independent lines of CBF2:: GUS transgenic plants were tested and produced similar results (data not shown).

Deletion analysis of the *CBF2* promoter identified a 125-bp region that is sufficient to impart cold-regulated gene expression when fused to the GUS reporter gene (Zarka et al., 2003). These sequences, which lie between −189 and −65 bp relative to the start of translation, are present in a 155-bp subfragment of the *CBF2* promoter that was used to test for circadian gating of cold responsiveness. The results indicate that, as with the entire *CBF2* promoter, the 155-bp subfragment fused to the GUS reporter (155::GUS) imparted cold-regulated gene expression and that the amplitude of the response was gated; there were peaks at ZT4, 28, and 52 and troughs at ZT16 and ZT40 (Fig. 4, C and D). The results also show that the amplitude of the response was much greater than what was observed with the entire *CBF2* promoter and similar to that of the endogenous *CBF2* transcript. These observations indicate that a promoter element(s) that confers gating of the cold response by the circadian clock is present in the same fragment of the *CBF2* promoter as are elements involved in cold induction.

Cold Responsiveness of *RAV1* and ZAT12 Are Gated by the Circadian Clock but in Opposite Phases

RAV1, which encodes an AP2/B3 domain transcription factor (Kagaya et al., 1999), and *ZAT12*, which

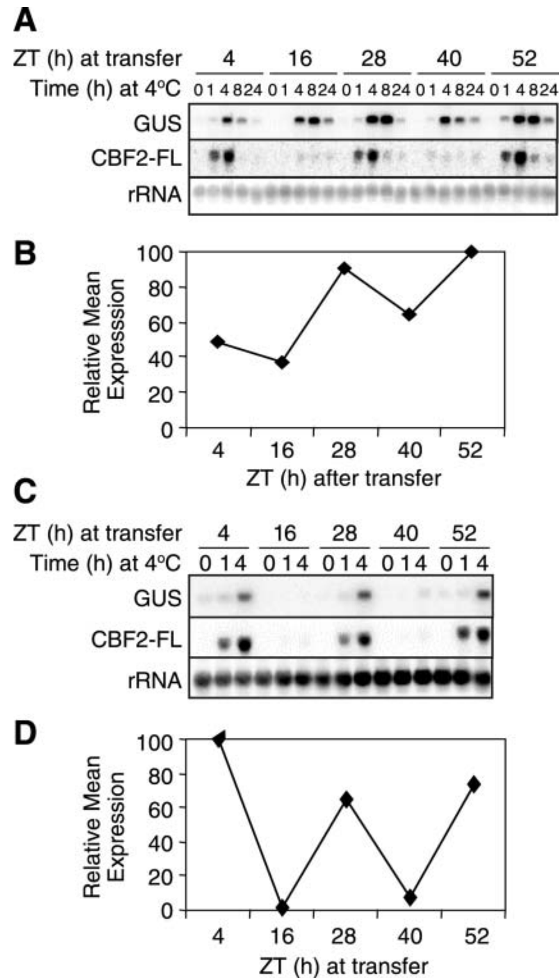

Figure 4. Circadian gating of *CBF1-3* cold responsiveness involves transcriptional regulation. Transgenic Arabidopsis plants, carrying the *GUS* reporter gene under the control of 1-kb and 155-bp fragments of the *CBF2* promoter, CBF2::GUS and 155::GUS, respectively (see text), were grown and treated as described in the legend for Figure 3. RNA gel blots, prepared from total RNA, were hybridized with probes for *GUS* and *CBF2*-FL. A, RNA gel blot showing cold responsiveness of the CBF2::GUS reporter gene. B, Graph of data from A showing mean expression of *GUS* at time points where cold induction of the *CBF* genes is maximal (1 and 4 h at 4°C). Mean expression levels are presented as a proportion of the highest value after normalization with respect to 25S rRNA expression levels. C, RNA gel blot showing cold responsiveness of the 155::GUS reporter gene. D, Graph of data from C showing mean expression of *GUS* at time points where cold induction of the *CBF* genes is maximal (1 and 4 h at 4°C). Mean expression levels are presented as a proportion of the highest value after normalization with respect to 25S rRNA expression levels. The subtle differences in the kinetics observed for cold-induced accumulation of the endogenous *CBF* and reporter *GUS* transcripts at ZT4, 28, and 52 are likely to involve differences in transcript stability and, potentially, differences in promoter activity.

encodes a zinc-finger domain transcription factor (Rizhsky et al., 2004), are up-regulated in parallel with *CBF1-3* in response to low temperature (Fowler and Thomashow, 2002). Moreover, *RAV1* transcript levels are circadian regulated under noninducing (warm) conditions with a similar phase to *CBF3* (Harmer et al., 2000). We therefore tested whether cold induction of *RAV1* and *ZAT12* was gated by the circadian clock in a similar fashion to *CBF1-3.* Northern-blot analysis of *RAV1* and *ZAT12* (using the RNA samples analyzed in Fig. 1) revealed that the maximal levels of *RAV1* transcript cycled in a manner similar to *CBF1-3* with higher cold-induced *RAV1* transcript accumulation occurring at ZT4 than at ZT16 (Fig. 5). In addition, cold-induced accumulation of *ZAT12* transcripts also cycled. However, in this case, the rhythmic pattern was approximately 180° out of phase with the rhythms of *CBF2* and *RAV1*, i.e. transcript accumulation was greater at ZT16 and ZT40 than at ZT4, ZT28, and ZT52 (Fig. 5).

Cold Induction of *CBF* Target Genes *COR78* and *COR6.6* Is Little Affected by the Circadian Clock

The *CBF1-3* transcriptional activators induce expression of a set of cold-responsive genes known as the CBF regulon (Fowler and Thomashow, 2002; Maruyama et al., 2004; Vogel et al., 2005). Given the effects of the clock on *CBF1-3* transcript accumulation, it was possible that cold-induced accumulation of transcripts for CBF regulon genes might also show gating that was regulated by the clock. To test this, we first probed the RNA samples shown in Figure 2A for *COR78* transcript levels but observed no cycling in the extent of cold-induced accumulation (data not shown). However, it was possible that peak and trough samples were missed with these particular time points, as Harmer et al. (2000) showed that peak level of *COR6.6* transcript accumulation in plants grown at constant warm temperature occurred at ZT12. Thus, to explore the issue further, Arabidopsis plants were entrained in a 12-h photoperiod, released into LL at ZT0, and then transferred to 4°C for 1, 4, and 24 h at 12-h intervals, beginning at ZT12, and the levels of *COR78* and *COR6.6* transcripts determined. The results (Fig. 6) again provided little evidence for cycling. If cycling did occur (there may be a hint of this suggested by the results), it was much less than that which occurred with the cold responsiveness of *CBF1-3*.

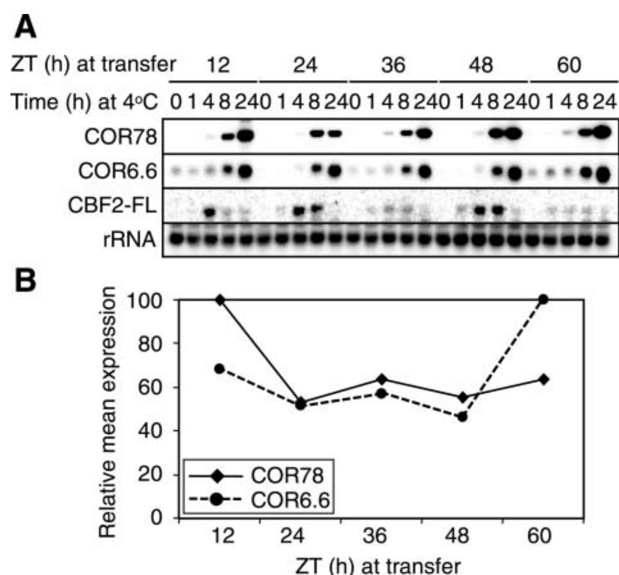

Figure 6. CBF target genes show attenuated circadian gating in response to low temperature. Wild-type Arabidopsis plants were grown and treated as described in the legend for Figure 3, except that plants were transferred to 4°C at 12-h intervals beginning at ZT12. A, RNA gel blots prepared from total RNA were hybridized with probes for *COR78, COR6.6*, and CBF2-FL. B, Graph of data from A showing mean expression of *COR78* and *COR6.6* at time points where these genes show induction by cold (4 h, 8, and 24 h at 4°C). Mean expression levels are presented as a proportion of the highest value for each gene after normalization with respect to 25S rRNA expression levels.

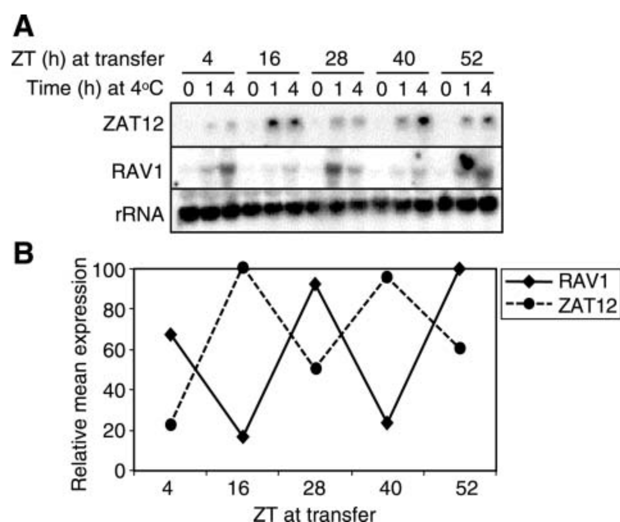

Figure 5. Cold induction of the *RAV1* and *ZAT12* genes is gated by the circadian clock. Wild-type Arabidopsis plants were grown and treated as described in the legend for Figure 3. A, RNA gel blots prepared from total RNA were hybridized with probes for *RAV1* and *ZAT12*. B, Graph of data from A showing mean expression of *RAV1* and *ZAT12* at time points where cold induction of these genes is maximal (i.e. 1 and 4 h at 4°C). Mean expression levels are presented as a proportion of the highest value after normalization with respect to 25S rRNA expression levels.

DISCUSSION

The CBF1-3 transcription factors induce expression of more than 100 genes known as the CBF (or DREB1) regulon (Fowler and Thomashow, 2002; Maruyama et al., 2004; Vogel et al., 2005). Expression of these genes leads to an increase in tolerance to freezing and chilling

temperatures (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Gong et al., 2002) as well as increased tolerance to drought and high salinity (Liu et al., 1998; Kasuga et al., 1999). Constitutive overexpression of the CBF regulon, however, can have negative effects on plant growth and development, including slow growth, reduced plant stature, delayed flowering, and reduced seed production (Liu et al., 1998; Kasuga et al., 1999; Gilmour et al., 2000). Thus, it is not surprising that regulation of the *CBF1-3* genes appears to include complex negative control. There is evidence that the thermometer that senses low temperature and provides signals to induce *CBF1-3* expression is desensitized by exposure to low temperature (Zarka et al., 2003); that *CBF2* (Novillo et al., 2004) and potentially downstream CBF regulon genes (Guo et al., 2002) comprise a negative regulatory loop that represses *CBF1-3* expression; and that the *CBF1-3* transcripts have a half-life of less than 10 min in plants at warm temperatures (Zarka et al., 2003), a turnover rate that is among the quickest documented for plant transcripts.

Another factor affecting expression of the *CBF* genes is the circadian clock. Harmer et al. (2000) have shown that *CBF3* transcripts accumulate to maximum levels in the early morning (ZT4) and reach minimum levels in the early evening (ZT16) in plants grown on a 12-h photoperiod at constant warm temperature. Here, we extend these findings to show that the circadian clock also gates expression of the *CBF1-3* genes in response to low temperature. The results presented indicate that the degree to which *CBF1-3* transcripts accumulate in response to low temperature is dependent on the phase of the clock; in early morning (ZT4) up-regulation is the greatest, and in early evening (ZT16) up-regulation is the least. In addition, the results of the promoter fusion experiments support the model that at least some of this regulation occurs at the transcriptional level, i.e. low temperature-induced *CBF2* promoter activity is gated by the clock. This was most apparent (Fig. 4) using a GUS reporter gene fusion regulated by a 155-bp subfragment of the *CBF2* promoter that contains two cold-regulatory elements, ICEr1 and ICEr2 (Zarka et al., 2003). Cycling also was observed with a CBF2::GUS fusion that included 1 kb of the *CBF2* promoter (and contained the 155-bp subfragment), though the amplitude of the cycling for this promoter fusion was considerably less than that observed using the 155-bp subfragment (Fig. 4). The reason for this difference remains to be determined, but may be due in part to the 1-kb element having cold-regulatory elements that are not subject to circadian regulation and are not present in the 155-bp fragment.

How might the clock gate low temperature-induced transcription of the *CBF1-3* genes? Many possibilities exist. The fact that the peak and trough of *CBF3* transcript accumulation in warm-grown plants occur at the same phases of the clock as do the greatest and least cold responsiveness of the *CBF1-3* genes raises the possibility that the two phenomena are mechanistically linked. For instance, the clock might affect the sensitivity of the thermometer that regulates *CBF1-3* expression. In the early morning, the clock could sensitize the thermometer to low temperature or, alternatively, in the evening it could desensitize the thermometer to low temperature. Thus, in the morning there would be a greater response of *CBF1-3* to low temperature than there would be in the evening. In addition, this clock-regulated sensitization/desensitization of the thermometer could also affect output from the thermometer at warm temperature and account for the cycling of *CBF3* transcript levels observed without a low-temperature stimulus. Of course, it is also possible that the mechanisms at work are independent. For instance, the promoters of the *CBF* genes could have both a regulatory element(s) that is responsive to the clock and a regulatory element(s) that is responsive to low temperature. In this model, the clock-regulated element might bind a repressor that is activated by the clock in the evening. At warm temperature, basal transcription from the *CBF1-3* promoters might occur at a low level in the morning, when the clock-regulated repressor is inactive, but not occur in the evening when the repressor is active. Moreover, exposing plants to low temperature in the morning would lead to full activation of the promoter without gating by the inactive clock-regulated repressor, whereas in the early evening, when the cold-regulated repressor is activated, low-temperature induction would be dampened.

Maruyama et al. (2004) recently concluded that the circadian clock has no effect on cold induction of *DREB1A* (*CBF3*), a conclusion that is in direct opposition to the conclusion that we draw here. In the experiments reported by Maruyama et al. (2004), Arabidopsis plants were grown under a 16:8-h photoperiod, whereas we used a 12:12-h photoperiod. However, we have also observed circadian-gated expression of the *CBF1-3* genes in Arabidopsis plants grown under a 16:8-h photoperiod and an 8:16-h photoperiod (S. Fowler, unpublished data). Thus, differences in photoperiod would not appear to offer an explanation for the different conclusions. One plausible explanation, however, regards the different time points used by Maruyama et al. (2004) and by us. Our results indicate that the peak and trough of *CBF1-3* responsiveness to cold induction occur at ZT4 and ZT16 (Fig. 2A) and that the differences in cold response would be considerably less at ZT6 and ZT12, the time periods used by Maruyama et al. (2004). In fact, close inspection of the results of Maruyama et al. (2004) hint that cold-induced accumulation of the *DREB1A* transcripts was slightly greater in the plants transferred to low temperature at ZT6 than at ZT12. Thus, the experimental results obtained by Maruyama et al. (2004) might not actually be in conflict with ours. Conclusively determining whether this is the case or whether subtle differences in environmental conditions can have a dramatic effect on the circadian gating of *CBF1-3* cold induction will require further experimentation.

In addition to *CBF1-3*, we found that the low-temperature responsiveness of *RAV1* and *ZAT12*, two

genes that are cold-induced with similar kinetics to *CBF1-3* (Fowler and Thomashow, 2002), was also gated by the clock. As with *CBF1-3*, the peak and trough of *RAV1* low-temperature responsiveness are at ZT4 and ZT16, respectively. By contrast, the peak and trough of *ZAT12* responsiveness are in opposite phase to *CBF1-3* and *RAV1*, occurring at ZT16 and ZT4, respectively. This finding is noteworthy, as expression of *ZAT12* has been shown to dampen expression of *CBF1-3* (Vogel et al., 2005). Thus, up-regulation of *ZAT12* in the evening could contribute to down-regulation of *CBF1-3* at night. In addition, the reverse phases of the gating for *CBF1-3* and *RAV1* versus *ZAT12* indicate that clock regulation of these genes may occur through at least two nonidentical (though potentially overlapping) pathways.

The clock-regulated gating of cold-induced expression of the *CBF1-3* genes has aspects in common with the regulation of Arabidopsis chlorophyll *a*/*b*-binding protein (*CAB*) genes. *CAB* gene expression is responsive to both light and the circadian clock (Millar and Kay, 1996). Clock-regulated expression of the *CAB* genes results in peak and trough transcript levels during subjective day and night, respectively, when plants are transferred from a 12-h photoperiod to LL or dark. Moreover, the magnitude of the light responsiveness of the *CAB* genes is high during the subjective day and very low during the subjective dark, i.e. the light responsiveness of the *CAB* genes is gated by the circadian clock. The precise mechanism for the circadian-regulated gating of the light responsiveness of the *CAB* genes is not known but involves action of the *EARLY FLOWERING 3* gene (Carré, 2002).

The results presented establish that the low-temperature responsiveness of *CBF1-3* is gated by the circadian clock. However, the biological significance of this regulation remains to be determined. The cold responsiveness of two CBF target genes, *COR78* and *COR6.6*, was, at most, marginally affected by the clock, raising the question of whether the gating of cold-induced accumulation of *CBF1-3* transcripts has a significant impact on the cold-regulated expression of genes downstream of *CBF1-3*. In addition, it seems counterintuitive that *CBF1-3* expression in the evening would be dampened by the clock as temperatures are generally lowest during the night. Perhaps this phasing reflects the delay between transcription of the *CBF* genes, synthesis of CBF protein, transcription of target genes, and production of target gene protein. Regardless, it is now clear that the circadian clock has at least two effects on *CBF* gene expression: a cycling of the transcript levels (and presumably protein levels) of *CBF3* in warm-grown plants and a gating of the cold-induced accumulation of *CBF1-3* transcripts. A better understanding of the molecular bases for this regulation should not only provide insight into the nature of the cold-signaling pathway responsible for *CBF1-3* gene expression, but may also shed new light on output pathways from the circadian clock.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) L. Heynh. ecotype Columbia (Col)-0 and transgenic plants in the Col background were grown in petri plates on Gamborg's B-5 medium (Life Technologies, Gaithersburg, MD) at pH 5.7 supplemented with 2% Suc and solidified with 0.8% phytagar (Life Technologies). The transgenic Arabidopsis plants used in this study were expressing a fusion of a 1-kb (CBF::GUS) or 155-bp (dimer, 155::GUS) fragment of the CBF2 promoter to the GUS reporter gene (Zarka et al., 2003) or constitutively expressing *CCA1* (*CCA1*-OX, Wang and Tobin, 1998). After stratification at 4°C for 3 d, seedlings were grown for 2 weeks in controlled environment chambers at 24°C with a 12-h light period under 80 to 100 μmol m^{-2} s^{-1} cool-white fluorescent illumination and 12-h dark period (12:12 photoperiod). Plants were transferred to low temperature (4°C) under 15 to 20 μmol m^{-2} s^{-1} cool-white fluorescent illumination at the ZT intervals indicated and harvested after various times of exposure to 4°C.

RNA Gel-Blot Hybridization Analysis

Total RNA was extracted from Arabidopsis plants using the RNeasy plant mini kit (Qiagen USA, Valencia, CA) as detailed by the manufacturer. Northern transfers were prepared, hybridized, and washed at high stringency as described by Stockinger et al. (1997). Gene-specific probes for *CBF1-3* were prepared as described previously (Gilmour et al., 1998), while a full-length *CBF2* probe, isolated from a cDNA clone encoding this gene, was used to detect expression of all three CBF genes simultaneously. Probes for *GUS* (Baker et al., 1994), *COR78*, *COR6.6* (Gilmour et al., 2000), *RAV1*, and *ZAT12* (Fowler and Thomashow, 2002) were prepared as described previously. To estimate relative loading and transfer, filters were hybridized a second time with probes for the constitutively expressed eukaryotic initiation factor 4A (*eIF-4A*) gene (Metz et al., 1992) or 25S rDNA (Delseny et al., 1983). Probes were labeled with ^{32}P using the Random Primers DNA labeling system (Invitrogen, Carlsbad, CA) as directed by the manufacturer. Membranes were exposed to a phosphorimager plate (Eastman-Kodak, Rochester, NY) and the image visualized by scanning the plate in a Fluor-S MultiImager (Bio-Rad Laboratories, Hercules, CA). Quantification was performed using Quantity One software, version 4.2.2 (Bio-Rad Laboratories).

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

ACKNOWLEDGMENTS

We thank Elaine Tobin for the gift of *CCA1*-OX seed, Rob McClung and Joel Kreps for helpful discussions about the circadian clock, Marc and Heather Knight for discussions about *CBF* gene regulation, and Sarah Gilmour for help in preparing the manuscript.

Received December 14, 2004; returned for revision December 30, 2004; accepted December 30, 2004.

LITERATURE CITED

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

Beator J, Kloppstech K (1992) The effect of heat-shock on morphogenesis in barley: coordinated circadian regulation of messenger-RNA levels for light-regulated genes and of the capacity for accumulation of chlorophyll protein complexes. Plant Physiol **100:** 1780–1786

Bünning E (1973) The Physiological Clock, Ed 3. Springer-Verlag, New York

Carpenter CD, Kreps JA, Simon AE (1994) Genes encoding glycine-rich *Arabidopsis thaliana* proteins with RNA-binding motifs are influenced by

cold treatment and an endogenous circadian rhythm. Plant Physiol **104:** 1015–1025

Carré IA (2002) ELF3: a circadian safeguard to buffer effects of light. Trends Plant Sci **7:** 4–6

Couderchet M, Koukkari WL (1987) Cold sensitivity oscillations of young soybean plants. Prog Clin Biol Res **227A:** 59–65

Delseny M, Cooke R, Penon P (1983) Sequence heterogeneity in radish nuclear ribosomal RNA genes. Plant Sci Lett **30:** 107–119

Devlin PF, Kay SA (2001) Circadian photoperception. Annu Rev Physiol **63:** 677–694

Eriksson ME, Millar AJ (2003) The circadian clock: a plant's best friend in a spinning world. Plant Physiol **132:** 732–738

Fowler S, Thomashow MF (2002) Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. Plant Cell **14:** 1675–1690

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

Green RM, Tobin EM (2002) The role of CCA1 and LHY in the plant circadian clock. Dev Cell **2:** 516–518

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

Harmer SL, Hogenesch JB, Straume M, Chang HS, Han B, Zhu T, Wang X, Kreps JA, Kay SA (2000) Orchestrated transcription of key pathways in Arabidopsis by the circadian clock. Science **290:** 2110–2113

Heintzen C, Melzer S, Fischer R, Kappeler S, Apel K, Staiger D (1994) A light- and temperature-entrained circadian clock controls expression of transcripts encoding nuclear proteins with homology to RNA-binding proteins in meristematic tissue. Plant J **5:** 799–813

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

Kagaya Y, Ohmiya K, Hattori T (1999) RAV1, a novel DNA-binding protein, binds to bipartite recognition sequence through two distinct DNA-binding domains uniquely found in higher plants. Nucleic Acids Res **27:** 470–478

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

Kloppstech K, Otto B, Sierralta W (1991) Cyclic temperature treatments of dark-grown pea seedlings induce a rise in specific transcript levels of light-regulated genes related to photomorphogenesis. Mol Gen Genet **225:** 468–473

Knight H, Zarka DG, Okamoto H, Thomashow MF, Knight MR (2004) Abscisic acid induces CBF gene transcription and subsequent induction of cold-regulated genes via the CRT promoter element. Plant Physiol **135:** 1710–1717

Kreps JA, Simon AE (1997) Environmental and genetic effects on circadian clock-regulated gene expression in Arabidopsis. Plant Cell **9:** 297–304

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

Maruyama K, Sakuma Y, Kasuga M, Ito Y, Seki M, Goda H, Shimada Y, Yoshida S, Shinozaki K, Yamaguchi-Shinozaki K (2004) Identification of cold-inducible downstream genes of the Arabidopsis DREB1A/CBF3 transcriptional factor using two microarray systems. Plant J **38:** 982–993

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

Metz AM, Timmer RT, Browning KS (1992) Sequences for two cDNAs encoding *Arabidopsis thaliana* eukaryotic protein synthesis initiation factor 4A. Gene **120:** 313–314

Michael TP, Salome PA, McClung CR (2003) Two Arabidopsis circadian oscillators can be distinguished by differential temperature sensitivity. Proc Natl Acad Sci USA **100:** 6878–6883

Millar AJ, Kay SA (1996) Integration of circadian and phototransduction pathways in the network controlling CAB gene transcription in Arabidopsis. Proc Natl Acad Sci USA **93:** 15491–15496

Mizoguchi T, Wheatley K, Hanzawa Y, Wright L, Mizoguchi M, Song HR, Carre IA, Coupland G (2002) LHY and CCA1 are partially redundant genes required to maintain circadian rhythms in Arabidopsis. Dev Cell **2:** 629–641

Novillo F, Alonso JM, Ecker JR, Salinas J (2004) CBF2/DREB1C is a negative regulator of CBF1/DREB1B and CBF3/DREB1A expression and plays a central role in stress tolerance in Arabidopsis. Proc Natl Acad Sci USA **101:** 3985–3990

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

Rikin A, Dillwith JW, Bergman DK (1993) Correlation between the circadian rhythm of resistance to extreme temperatures and changes in fatty acid composition in cotton seedlings. Plant Physiol **101:** 31–36

Rizhsky L, Davletova S, Liang H, Mittler R (2004) The zinc finger protein Zat12 is required for cytosolic ascorbate peroxidase 1 expression during oxidative stress in Arabidopsis. J Biol Chem **279:** 11736–11743

Schaffer R, Ramsay N, Samach A, Corden S, Putterill J, Carre IA, Coupland G (1998) The late elongated hypocotyl mutation of Arabidopsis disrupts circadian rhythms and the photoperiodic control of flowering. Cell **93:** 1219–1229

Smallwood M, Bowles DJ (2002) Plants in a cold climate. Philos Trans R Soc Lond B Biol Sci **357:** 831–847

Somers DE, Webb AA, Pearson M, Kay SA (1998) The short-period mutant, *toc1-1*, alters circadian clock regulation of multiple outputs throughout development in *Arabidopsis thaliana*. Development **125:** 485–494

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

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

Vogel JT, Zarka DG, Van Buskirk HA, Fowler SG, Thomashow MF (2005) Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of Arabidopsis. Plant J **41:** 195–211

Wang ZY, Kenigsbuch D, Sun L, Harel E, Ong MS, Tobin EM (1997) A Myb-related transcription factor is involved in the phytochrome regulation of an Arabidopsis Lhcb gene. Plant Cell **9:** 491–507

Wang ZY, Tobin EM (1998) Constitutive expression of the *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) gene disrupts circadian rhythms and suppresses its own expression. Cell **93:** 1207–1217

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

Zarka DG, Vogel JT, Cook D, Thomashow MF (2003) 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. Plant Physiol **133:** 910–918