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

Sarah G. Fowler², Daniel Cook²,³, 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; Small-wood 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 over-expressing 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, COR15A 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...
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](image-url). 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.
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 at 4, 16, 24, 32, and 40 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 elf4a 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.

![Circadian Gating of CBF1-3 Cold Responsiveness Involves Transcriptional Regulation](image)

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 (GLI5) reporter gene under the control of a 1-kb fragment of the CBF2 promoter (CBF2::GLU5). This gene fusion had previously been shown to be cold regulated (Zarka et al., 2003). After
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...
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.

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 Thomashowa, 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. 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 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- kv (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 CAI (CIA-1-OX, Wang and Tobin, 1996). 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, COR8.6 (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 ukyarotic initiation factor 4A (CIF-4A) gene (Metz et al., 1992) or 25S rDNA (Delseny et al., 1983). Probes were labeled with 32P 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 Multilmager (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**


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


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


