# CIRCADIAN CLOCK-ASSOCIATED 1 and LATE ELONGATED HYPOCOTYL regulate expression of the C-REPEAT BINDING FACTOR (CBF) pathway in *Arabidopsis*

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**The C-REPEAT BINDING FACTOR (CBF) cold-response pathway has a prominent role in cold acclimation, the process whereby certain plants increase tolerance to freezing in response to low nonfreezing temperatures. In *Arabidopsis*, the CBF pathway is characterized by rapid induction of the *C-REPEAT BINDING FACTOR 1* (*CBF1*), *CBF2*, and *CBF3* genes, which encode transcriptional activators, followed by induction of the CBF-targeted genes known as the "CBF regulon." Expression of the CBF regulon results in an increase in freezing tolerance. Previous studies established that *CBF1*, *CBF2*, and *CBF3* are subject to circadian regulation and that their cold induction is gated by the circadian clock. Here we present the results of genetic analysis and ChIP experiments indicating that both these forms of regulation involve direct positive action of two transcription factors that are core components of the clock, i.e., CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY). In plants carrying the *cca1*-11/*lhy*-21 double mutation, cold induction of *CBF1*, *CBF2*, and *CBF3* was greatly impaired, and circadian regulation of *CBF1* and *CBF3* was essentially eliminated; circadian regulation of *CBF2* continued, although with significantly reduced amplitude. Circadian regulation and cold induction of three CBF regulon genes, i.e., *COLD-REGULATED GENE15A* (*COR15A*), *COR47*, and *COR78*, also were greatly diminished in plants carrying the *cca1*-11/*lhy*-21 double mutation. Furthermore, the *cca1*-11/*lhy*-21 double mutation resulted in impaired freezing tolerance in both nonacclimated and cold-acclimated plants. These results indicate that CCA1/LHY-mediated output from the circadian clock contributes to plant cold tolerance through regulation of the CBF cold-response pathway.**

A general feature of plants from temperate environments is that they increase in freezing tolerance in response to low nonfreezing temperatures, a process called "cold acclimation" (1, 2). It now is well established that cold acclimation involves extensive changes in gene expression (3–6). The best understood cold-regulatory pathway is the CBF pathway. This pathway, which is widely conserved in plants (7), is best characterized in *Arabidopsis* (8, 9). When *Arabidopsis* plants are transferred from warm to cold temperature, *C-REPEAT BINDING FACTOR 1* (*CBF1*), *2* (*CBF2*), and *3* (*CBF3*)—also known as *DROUGHT RESPONSE ELEMENT BINDING FACTOR 1B* (*DREB1B*), 1C (*DREB1C*), and 1A (*DREB1A*), respectively—are induced rapidly. These genes, which are linked physically in tandem array, encode transcription factors that are members of the AP2/ERF family of DNA-binding proteins (10). The CBF proteins bind to the CRT/DRE regulatory element present in the promoters of about 100 cold-regulated (COR) genes, known as the "CBF regulon," and induce their expression (4, 6, 11). Constitutive overexpression of *CBF1, CBF2*, and *CBF3* at warm temperature results in constitutive expression of the CBF regulon and an increase in freezing tolerance (12–14). The mechanisms whereby expression of the CBF regulon promotes freezing tolerance are not completely understood but involve the synthesis of low molecular weight cryoprotectants such as sucrose and raffinose and proteins that have cryoprotective properties (1, 2).

Given their importance in cold acclimation, efforts have been directed at understanding the mechanisms involved in cold-induction of *CBF1, CBF2*, and *CBF3*. To date, two positive regulators have been identified: INDUCER OF CBF EXPRESSION 1 (ICE1), a Myc family transcription factor that positively regulates *CBF3* (15), and CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR 3 (CAMTA3), a CAMTA family transcription factor that positively regulates *CBF1* and *CBF2* (16). The *ICE1* and *CAMTA3* genes are transcribed at warm temperature, indicating that their activities involve posttranscriptional regulatory mechanisms that are responsive to low temperature (16–18).

Another factor that affects the expression of *CBF1, CBF2*, and *CBF3* is the circadian clock (19–22). At warm temperature, the transcript levels for *CBF1, CBF2*, and *CBF3* oscillate with a peak at about 8 h after dawn (zeitgeber time 8; ZT8) and a trough at about ZT20. Moreover, cold-induction of *CBF1, CBF2*, and *CBF3* is "gated" by the clock (22); if plants are exposed to low temperature at ZT4, the increase in *CBF1, CBF2*, and *CBF3* transcript levels is much greater than if plants are exposed to low temperature at ZT16. These results indicate that cold induction of *CBF1, CBF2* and *CBF3* involves the integration of low-temperature and clock-regulatory pathways.

The circadian clock of *Arabidopsis* consists of multiple interlocking regulatory feedback loops (23, 24). Key components of the core feedback loop are CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), Myb transcription factors that have partially overlapping functions (25–28), and TIMING OF CAB 1 (TOC1), a PSEUDO RESPONSE REGULATOR (PRR) protein (29). Expression of *CCA1* and *LHY* peaks just after dawn, whereas the expression of *TOC1* peaks in the early evening. CCA1 and LHY bind to the Evening Element (EE) (19) present in the promoter of *TOC1* and repress its transcription (30). TOC1 is necessary for the induction of both *CCA1* and *LHY* (28). TOC1 is known to inhibit the repression of *CCA1* by the TCP transcription factor CCA1 HIKING EXPEDITION 1 (CHE1) (31), but the means by which *LHY* expression is activated by TOC1 remains unknown. CCA1 and LHY also regulate expression of *PSEUDO RESPONSE REGULATORS 7* (*PRR7*) and *9* (*PRR9*) (32, 33), two components of the morning regulatory loop. CCA1 and LHY bind to the promoters of these two genes to induce their expression, and the PRR7 and PRR9 proteins then negatively regulate *CCA1* and *LHY* (32, 33).

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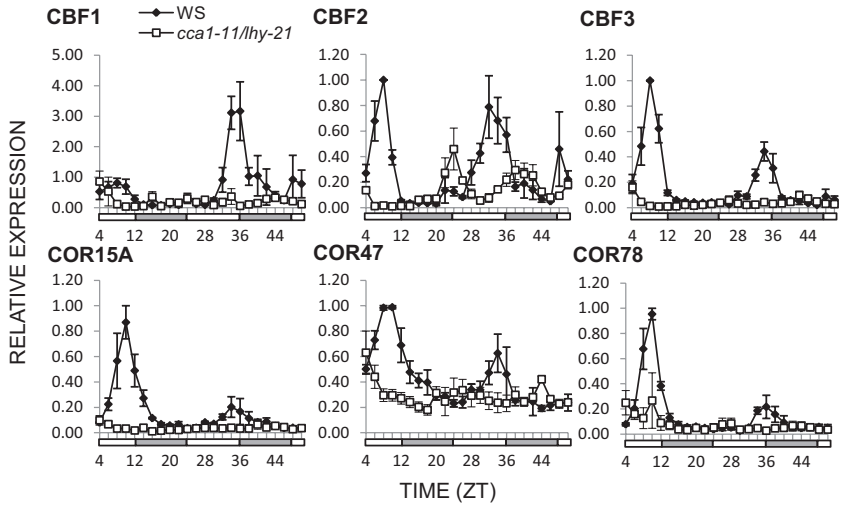


**Fig. 1.** Effects of the *cca1-11/lhy-21* double mutation on circadian regulation of *CBF1*, *CBF2*, *CBF3*, and CBF-targeted genes *COR15A*, *COR47*, and *COR78*. Wild-type Wassilewskija-2 (WS) and *cca1-11/lhy-21* double-mutant plants were grown at 22 °C under a 12-h photoperiod to the four-leaf stage and then were transferred to constant light at zeitgeber time 0 (ZT0) (subjective day and night are indicated by white and gray bars, respectively). Plants were harvested every 2 h, and the transcript levels for the indicated genes were determined by qRT-PCR. Gene expression was normalized to *ISOPENTENYL PYROPHOSPHATE (IPP2)* for each sample. Gene expression is relative to one wild-type sample set to a value of 1 for each biological replicate. Values are averages from three independent biological experiments. Error bars indicate SEM.

It was reported recently that *Arabidopsis* plants carrying the *prr5*/*prr7*/*prr9* triple mutation constitutively express *CBF1*, *CBF2*, and *CBF3* at high levels and display constitutively high levels of freezing tolerance (34). Thus, it was proposed that PRR5, PRR7, and PRR9 might act as direct negative regulators of *CBF1*, *CBF2*, and *CBF3* (34). Here we present results indicating that the clock also provides positive regulation of the CBF cold-response pathway and enhances freezing tolerance through action of the core clock components CCA1 and LHY.

## Results

**CCA1 and LHY Have a Direct Role in Circadian Regulation of *CBF1*, *CBF2*, and *CBF3*.** Consistent with previous reports (19–21), we found that *CBF1, CBF2*, and *CBF3* are subject to circadian regulation (Fig. 1). Transcript levels for *CBF1, CBF2*, and *CBF3* oscillated with a peak occurring at about ZT8 followed by a second peak about 24 h later. The oscillation patterns for the three *CBF* genes were similar, although we observed one consistent difference; whereas the ZT8 peak for *CBF1* was lower than the second peak, the ZT8 peaks for *CBF2* and *CBF3* were higher than the second peak. Thus, the transition from dark to light may have a specific effect on the regulation of *CBF1*.

Three lines of evidence led us to think that CCA1 and LHY might drive circadian regulation of *CBF1, CBF2*, and *CBF3*. First, the protein levels for CCA1 and LHY peak in the early morning (ZT1–3) (26, 35, 36), just before the time that the transcript levels of *CBF1, CBF2,* and *CBF3* begin to increase. Second, the transcript levels for *PRR7* (32, 36) and *LIGHT-HARVESTING COMPLEX B* (*LHCB*) (24, 35, 37), both of which are induced by CCA1 and LHY, peak similarly to the *CBF* genes. Last, the promoter regions of *CBF1, CBF2*, and *CBF3* have several EE (AAAATATCT) (19) and CCA1-binding sites (CBS; AATCT) (35) (Fig. 2) that mediate binding of CCA1 and LHY (30, 35, 38) to target promoters. To determine whether CCA1 and LHY were involved in circadian regulation of *CBF1*, *CBF2*, and *CBF3*, we asked whether their expression was affected in plants carrying either the single *cca1-11* or *lhy-21* null mutations or the *cca1-11*/*lhy-21* double mutation. We found that the single mutations had dif-

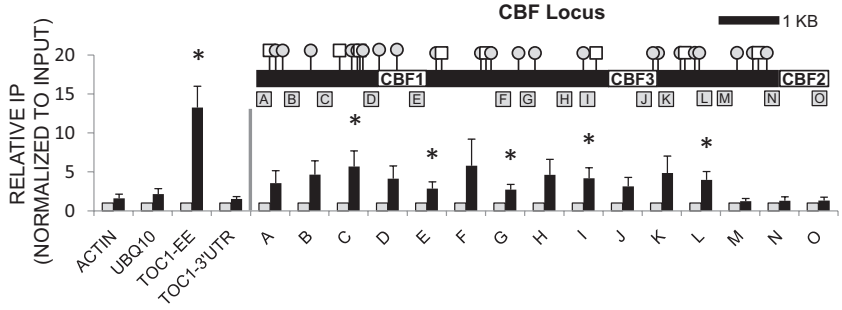


**Fig. 2.** Binding of CCA1 at the *CBF1-3* locus. *cca1-1* and *cca1-1 CCA1p:CCA1-GFP* plants were grown at 22 °C under a 12-h photoperiod to the four-leaf stage. Tissue was fixed at ZT4, and ChIP was performed using anti-GFP antibody. Immunoprecipitated DNA was quantified by qRT-PCR using primers specific to regions within the *CBF1-3* locus (boxes A through O). The levels of immunoprecipitated DNA were normalized to the respective input DNA. Immunoprecipitation in *cca1-1 CCA1p:CCA1-GFP* plants (black bars) is relative to *cca1-1* plants (gray bars) set to a value of 1. Primer pairs directed to the 3′ UTR of *TOC1* (TOC1-3′UTR), *ACTIN* 7, and *UBIQUITIN 10* (*UBQ10*) were used as negative controls (31). Primers near the EE element in the *TOC1* promoter (TOC1-EE) were used as a positive control. Values represent the average of five independent biological experiments. Error bars indicate SEM. *$P < 0.05$ using a paired, one-tailed *t* test. In the *CBF* locus diagram, the transcribed regions are indicted by white boxes, and the approximate positions of CBS [$(A_{2-4})$TCT], and EE (AAAATATCT) motifs are indicated by gray circles and white squares, respectively.

fering effects on the three *CBF* genes, whereas cycling of *CBF1* was severely disrupted, *CBF2* and *CBF3* transcript levels cycled with approximately the same amplitudes as in the wild-type plants, although the peaks occurred about 2 h earlier than in the wild-type plants (Fig. S1). Period shortening of output genes such as *LHCB* has been observed previously in *cca1* and *lhy* mutant plants (39, 40) as well as in our experiments (Fig. S2). The *cca1-11/lhy-21* double mutations also had differing effects on the circadian regulation of the three *CBF* genes (Fig. 1); circadian regulation of *CBF1* and *CBF3* was essentially eliminated in the double-mutant plants, but *CBF2* transcript levels clearly continued to cycle, although the amplitude was diminished, and the period was greatly shortened. Period shortening of output genes such as *LHCB* also has been observed previously in *cca1-11/lhy-21* double-mutant plants (40, 41) and in our experiments (Fig. S3). From these results we concluded that circadian regulation of *CBF1* and *CBF3* is dependent on the action of either CCA1 or LHY and that circadian regulation of *CBF2* involves action of CCA1 and LHY but can be driven to a considerable degree by other unknown factors.

CCA1 and LHY may impart circadian regulation of *CBF1, CBF2*, and *CBF3* by binding to the EE and CBS motifs present in the *CBF* promoters and act as positive regulators stimulating transcription. To test this hypothesis, we conducted ChIP experiments to determine whether CCA1 binds directly to the promoter regions of *CBF1, CBF2*, and *CBF3*. To do so, we compared ChIP results obtained with plants carrying the *cca1-1* mutation and *cca1-1* plants that had been restored with a construct encoding the CCA1 protein tagged with GFP under the endogenous CCA1 promoter (31). Chromatin was isolated from plants harvested at ZT4, the point when *CBF* transcript levels begin to rise. In mock experiments where rabbit IG was used for precipitation, no specific binding was detected for any of the subregions (A to O in Fig. S4) of the *CBF1-3* locus tested or for *TOC1*, *ACTIN 7*, or *UBIQUITIN 10* (Fig. S4). In contrast, test experiments indicated that specific binding of the CCA1-GFP protein occurred throughout most of the *CBF1-3* locus and also within the promoter region of *TOC1*, a positive control, but not in the promoters of *ACTIN 7* or *UBIQUITIN 10*, two negative controls (31) (Fig. 2). Significant CCA1-GFP binding occurred in the promoter regions of *CBF1*, *CBF2*, and *CBF3* (C, L, and G and I, respectively in Fig. 2). We also observed CCA1 associated with the coding region of *CBF1* (E in Fig. 2), perhaps because of the tandem connection of the CBF genes and consequently close proximity to several CCA1 binding sites located in the adjacent *CBF3* promoter (Fig. 2). Thus, although it is possible that there is CCA1 binding within the *CBF1* transcript region, the actual binding site could be downstream in the *CBF3* promoter. In sum, the results of our genetic and ChIP experiments support the model that circadian regulation of *CBF1, CBF2*, and *CBF3* involves action of CCA1 and LHY binding to the promoters of these genes and up-regulating their transcription during the morning hours.

**Rhythmic Expression of CBF Regulon *COR* Genes and Freezing Tolerance Are Impaired in Plants Carrying the *cca1*-11/*lhy*-21 Double Mutation.** Harmer et al. (19) suggested that the circadian regulation of *CBF1*, *CBF2*, and *CBF3* could result in rhythmic expression of CBF regulon *COR* genes. To test this possibility, we examined the transcript levels for three CBF-inducible genes—*COR15A*, *COR47*, and *COR78*—in wild-type plants and in plants carrying the *cca1-11/lhy-21* double mutation. The results indicated that the transcript levels for all three *COR* genes oscillated with a period of about 24 h in wild-type plants, although for *COR15A* and *COR78* the amplitude of the second peak was much less than that of the first peak (Fig. 1). For all three genes, the first peak occurred at about ZT10, consistent with the transcript levels of *CBF1*, *CBF2*, and *CBF3* peaking just before this time, at about ZT8 (19, 20) (Fig. 1). Moreover, the oscillation in *COR* transcript levels was largely reduced in the *cca1-11/lhy-21* double-mutant plants (Fig. 1). These results were consistent with the model that circadian-regulated expression of *CBF1*, *CBF2*, and *CBF3* imparts rhythmic expression of CBF-targeted *COR* genes at "basal" nonacclimating temperatures.

The decrease in expression of the CBF-targeted *COR* genes in the *cca1-11/lhy-21* double-mutant plants could result in a decrease in basal freezing tolerance. We tested this possibility by using the electrolyte leakage assay to compare the freezing tolerance of wild-type and *cca1-11/lhy-21* plants. The results indicated that the *cca1-11/lhy-21* double mutation reduced freezing tolerance by about 50%; although the temperature at which cell damage results in release of 50% of total electrolytes ($EL_{50}$) was about −4 °C in wild-type plants, it was about −2 °C in the *cca1-11/lhy-21* mutant plants (Fig. 3). Thus, the circadian clock is required for maximum basal freezing tolerance.

**CCA1 and LHY Regulate Cold Induction of *CBF1*, *CBF2*, and *CBF3*.** Fowler et al. (22) reported that cold induction of *CBF1*, *CBF2*, and *CBF3* is gated by the circadian clock. Given our results indicating a role for CCA1 and LHY in the circadian regulation of *CBF1, CBF2*, and *CBF3*, we asked whether these transcription factors also had a role in the gating phenomenon. As previously reported (22, 34), cold-induction of *CBF1*, *CBF2*, and *CBF3* was much greater in the subjective day than in the subjective evening (Fig. 4). This cold induction was little affected by the single *cca1-11* and *lhy-21* mutations (Fig. S1) but was greatly reduced in the *cca1-11/lhy-21* double mutants, and the period of cycling was shortened (Fig. 4), as was the period of cycling for *LHCB* (Fig. S3). Thus,

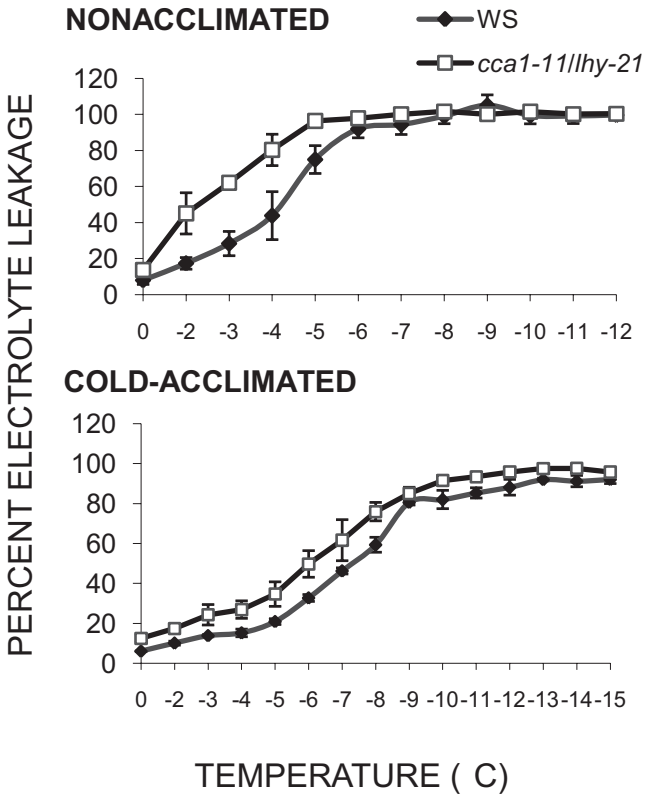


**Fig. 3.** Effect of the *cca1-11/lhy-21* double mutation on plant freezing tolerance. *cca1-11/lhy-21* double-mutant and wild-type Ws-2 (WS) plants were grown at 22 °C under a 12-h photoperiod for ~18 d and were tested directly for freezing tolerance (nonacclimated plants; *Upper*) or were transferred at ZT4 to 4 °C for 7 d under a 12-h photoperiod and then tested for freezing tolerance (cold-acclimated plants; *Lower*). Freezing tolerance was tested using the electrolyte leakage test. The results presented are average values from three independent experiments. Error bars indicate SEM.

CCA1 and LHY have a major role in the induction of *CBF1*, *CBF2*, and *CBF3* expression in response to low temperature.

**Cold induction of CBF-Targeted *COR* Genes and Freezing Tolerance Are Impaired in Plants Carrying the *cca1-11/lhy-21* Double Mutation.** The finding that cold induction of *CBF1*, *CBF2*, and *CBF3* was impaired in plants carrying the *cca1-11/lhy-21* double mutation prompted us to determine whether the double mutation also impaired cold induction of CBF-targeted *COR* genes and the freezing tolerance of cold-acclimated plants. In wild-type plants, the degree to which *COR15A*, *COR47*, and *COR78* were induced by low temperature cycled, with peaks in the late day and troughs in the late subjective evening (Fig. 4), times that were consistent with the cycling of *CBF1*, *CBF2*, and *CBF3* cold induction (Fig. 4). In plants carrying the *cca1-11/lhy-21* double mutation, the peak following the night-to-day transition was little affected, but the subsequent peaks were greatly diminished, and the period of cycling was shortened (Fig. 4). In addition, the freezing tolerance of cold-acclimated plants carrying the *cca1-11/lhy-21* double mutation was about 1 °C less than that of cold-acclimated wild-type plants (Fig. 3). These results indicate that CCA1 and LHY are required for *Arabidopsis* plants to attain maximum levels of *COR* gene induction and freezing tolerance in response to low temperature.

## Discussion

The CBF cold-response pathway is highly conserved among plants and has a major role in plant freezing tolerance (1–3, 9). Accordingly, there is considerable interest in understanding the mechanisms that control expression of this stress-response pathway. Here we establish that the CBF pathway is subject to positive regulation by the circadian clock components CCA1 and LHY. We show that these factors have roles in both circadian regulation and cold induction of the pathway and that they are required for plants to attain maximum freezing tolerance at both basal and cold-acclimating temperatures.

At basal growth temperature, the transcript levels for *CBF1, CBF2*, and *CBF3* oscillate, with peaks and troughs occurring at about ZT8 and ZT20, respectively (19–21) (Fig. 1). Our genetic and ChIP analyses indicate that this circadian regulation is caused by the direct action of CCA1 and LHY binding at the *CBF1-3* locus—presumably at the EE, CBS, and related motifs—and induces transcription of the *CBF* genes. In the morning hours, when CCA1 and LHY protein levels peak (25, 35, 36), the transcript levels of *CBF1, CBF2*, and *CBF3* peak; in the evening hours, when CCA1 and LHY protein levels are low, the transcript levels for *CBF1, CBF2*, and *CBF3* are low (Fig. 5). The finding that circadian regulation of *CBF1* and *CBF3* is nearly eliminated in plants carrying the *cca1-11/lhy-21* double mutation indicates that no other regulatory proteins are sufficient to impart positive circadian regulation of these genes. In contrast, circadian regulation of *CBF2* continues in plants carrying the *cca1-11/lhy-21* double mutation, albeit with reduced amplitude and shortened periodicity. Thus, at least one additional regulatory protein appears to drive positive circadian regulation of *CBF2*. Prime candidates for this residual regulation are the four REVEILLE (RVE) proteins RVE1, RVE3, RVE4, and RVE8 (42). These Myb-like transcription factors fall into the CCA1 subfamily, bind to the EE motif, are circadian regulated, and, like CCA1 and LHY, have peak transcript levels at dawn (42).

Kidokoro et al. (21) reported that circadian regulation of *CBF1*, *CBF2*, and *CBF3* also involves negative regulation. These investigators found that PHYTOCHROME INTERACTING FACTOR 7 (PIF7) binds to a G-box element in the promoter of *CBF2* and that this element is required for down-regulation of the *CBF2* promoter during the subjective evening. In addition, they found that PIF7 physically interacts with TOC1 (21). Thus, circadian-controlled down-regulation of the *CBF* genes appears to involve action of a PIF7–TOC1 protein complex binding to G-box elements in their promoters (Fig. 5).

In addition to establishing a role for CCA1 and LHY in circadian regulation of *CBF1*, *CBF2*, and *CBF3,* our results indicate that CCA1 and LHY also act as positive regulators of *CBF* cold induction. This action is evidenced by the finding that cold induction of *CBF1*, *CBF2*, and *CBF3* is greatly impaired in plants carrying the *cca1-11/lhy-21* double mutation (Fig. 4). We propose that the gating of *CBF1*, *CBF2*, and *CBF3* cold induction results, in part, from positive synergistic interaction between cold-signaling and clock-output pathways, the former mediated by ICE1 and CAMTA3 and the latter by CCA1 and LHY (Fig. 5). If the

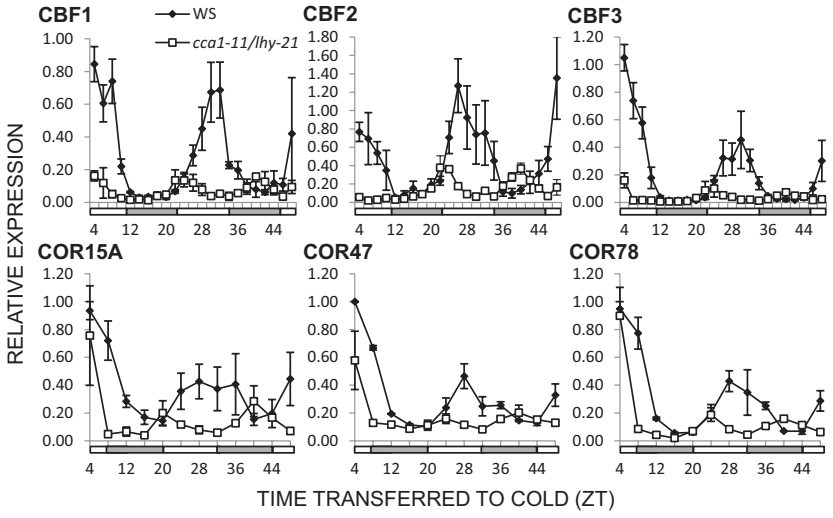


**Fig. 4.** Effects of the *cca1-11/lhy-21* double mutation on cold induction of *CBF1*, *CBF2*, *CBF3*, and CBF-targeted genes *COR15A*, *COR47*, and *COR78*. Wild-type Ws-2 (WS) and *cca1-11/lhy-21* double-mutant plants were grown at 22 °C under a 12- h photoperiod to the four- leaf stage and then were transferred to constant light at ZT0 (subjective day and night are indicated by white and gray bars, respectively). Plants were transferred to cold temperature (4 °C) for 2 h, every 2 h (*CBF* genes) or for 4 h every 4 h (*COR* genes) at the start of constant-light conditions. Transcript levels for the indicated genes were determined by qRT-PCR. The *x* axis represents the time (ZT) when plants were transferred to cold temperature. Gene expression was normalized to UBQ10 for each sample. Gene expression is relative to one wild-type sample set to a value of 1 for each biological replicate. Values are averages from three independent biological experiments. Error bars indicate SEM. Primer pair sequences are given in Table S1.

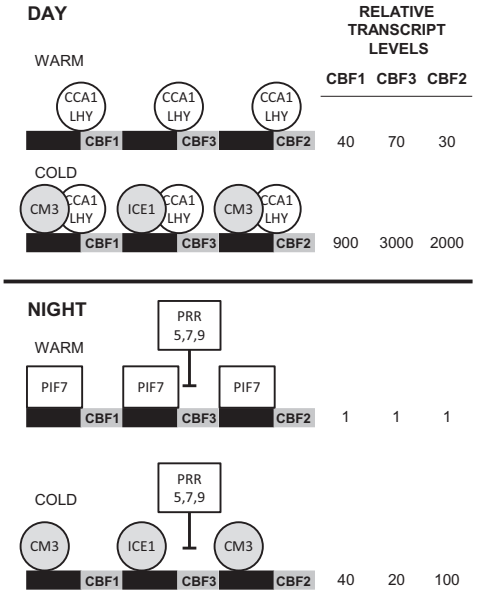


**Fig. 5.** Model for circadian regulation and gated cold induction of *CBF1*, *CBF2*, and *CBF3*. During the day, CCA1 and LHY bind throughout the *CBF* locus and promote *CBF* transcription. In the evening, CCA1 and LHY are at low levels and have little effect on *CBF* expression. Oscillations in CCA1 and LHY binding at the *CBF* locus largely account for the circadian regulation of the *CBF* genes. Circadian regulation also involves repression in the evening hours mediated by PIF7 (21) and PRR5, PRR7, and PRR9 (34). Transfer of plants to low temperature in the day or evening results in activation of ICE1 (15), CAMTA3 (CM3) (16), and possibly other transcription factors that stimulate transcription of the *CBF* genes. If the temperature drops during the day, the clock and cold-signaling pathways act synergistically to induce *CBF* expression to high levels. If plants are exposed to cold temperatures in the evening, there is no positive synergy between the two pathways, and there is repression by PRR5, PRR7, and PRR9 (34) leading to *CBF* induction at moderate levels. Relative transcript levels were calculated using peak and trough values obtained for the *CBF* genes in the experiments presented in Figs. 1 and 4 (the values obtained for plants grown at warm temperature and harvested in the evening were set to 1). See *Discussion* for details.

temperature drops in the morning, CCA1 and LHY are present at the *CBF* locus and can act with ICE1 and CAMTA3 to induce high-level expression of C*BF1*, *CBF2*, and *CBF3*. In contrast, if the temperature drops in the evening, CCA1 and LHY are at low levels; consequently there is little synergy between the low-temperature and clock pathways, and the induction of *CBF1*, *CBF2*, and *CBF3* is low, approximating the peak levels obtained with circadian regulation (Fig. 5).

Cold induction of *CBF1*, *CBF2*, and *CBF3* during the evening hours also may involve negative regulation. Such regulation would not appear to involve PIF7, because Kidokoro et al. (21) showed that the gating of *CBF1*, *CBF2*, and *CBF3* expression is not impaired in plants carrying the *pif7*-2 mutation (21). However, Nakamichi et al. (34) found that circadian regulation of *CBF1*, *CBF2*, and *CBF3* and the gating of their cold induction do not occur in plants carrying the *prr9*-11/*prr7*-10/*prr5*-10 triple mutation. When plants were grown at basal temperature, the transcript levels for *CBF1*, *CBF2*, and *CBF3* remained high throughout the day in the triple-mutant plants (34). Similarly, the cold induction of *CBF1*, *CBF2*, and *CBF3* in the triple-mutant plants remained at about the peak levels observed in wild-type plants regardless of the time of day at which the mutant plants were exposed to low temperature (34). Nakamichi et al. (34) concluded that PRR9, PRR7, and PRR5 are negative regulators of *CBF1*, *CBF2*, and *CBF3* and proposed two possible mechanisms. One is that PRR9, PRR7, and PRR5 directly repress expression of the *CBF* genes. Alternatively, they suggested that aberrant expression of the *CBF* genes might result from the "circadian disorder" caused by the *prr9*-11/*prr7*-10/*prr5*-10 triple mutation. Our results provide no direct evidence in favor of or against the first model. However, the consistently high *CBF* expression may be explained in part by the constitutively elevated expression of *CCA1* and *LHY* in the triple-mutant plants (34, 43).

A final point should be mentioned in regard to the role of the clock in freezing tolerance. Our results indicate that CCA1 and LHY are required for *Arabidopsis* to attain maximum levels of freezing tolerance at both non-acclimating and cold-acclimating temperatures (Fig. 3). Recently, Espinoza et al. (44) independently reached the same conclusion; they too found that the *cca1-11*/*lhy-21* double mutation resulted in impaired freezing tolerance. Our results also indicate a mechanism whereby cold-signaling and clock-regulatory pathways are integrated to condition freezing tolerance: the positive regulation of the CBF cold-response pathway mediated through CCA1 and LHY binding at the *CBF1–3* locus and inducing expression of *CBF1*, *CBF2*, and *CBF3* (Fig. 5). Taken together, our results suggest that the integration of cold-signaling pathways with the circadian clock may have been an important evolutionary event that has contributed to plant adaptation to cold environments.

## Materials and Methods

**Plant Material and Growth Conditions.** *Arabidopsis thaliana* ecotype Ws-2 and mutants in this background were grown as described previously (16). Homozygous T-DNA mutant lines were obtained from the *Arabidopsis* Biological Resource Center (45). Null mutations were checked by quantitative RT-PCR (qRT-PCR). These lines were *cca1-11*(CS9378), *lhy-21* (CS9379), and *cca1-11*/*lhy-21*(CS9380). Restored *cca1-1* line, CCA1p:CCA1-GFP under the CCA1 endogenous promoter and *cca1-1* (31), used in ChIP experiments, were generously donated by the Kay Laboratory (University of California, San Diego, La Jolla, CA).

All seeds were stratified for 3–5 d in the dark at 4 °C. Except for freezing-tolerance tests, plants were grown at 22 °C under sterile conditions on Gamborg's B5 medium (Caisson Laboratories) without sucrose at ~100 μmol $m^{-2}$ $s^{-1}$ in a 12-h photoperiod. For circadian experiments, plants were sampled at 22 °C in 100 μmol $m^{-2}$ $s^{-1}$ constant light or at 4 °C in 35 μmol $m^{-2}$ $s^{-1}$ constant light. For electrolyte leakage experiments, plants were grown as described (16) at ~100 μmol $m^{-2}$ $s^{-1}$ under a 12-h photoperiod. Cold- temperature treatment for plants grown on soil was at 4 °C in light at 35 μmol $m^{-2}$ $s^{-1}$ under a 12-h photoperiod.

**RNA Analysis.** RNA extraction was performed as described in ref. 16. For qRT-PCR (Applied Biosystems 7500 FAST Real-Time PCR System in FAST mode), cDNA was made as described in ref. 16, except that total RNA of either 0.2 or 0.025 μg was used for a 40-μL reverse-transcription reaction. In the 10-μL PCR reactions, 2 μL of diluted cDNA was used. *UBQ10* or *IPP2* were used as reference genes. All primer sets are listed in Table S1.

**ChIP.** ChIP experiments were carried out as described by Pruneda-Paz et al. (31) with a few modifications. CCA1p:CCA1-GFP and *cca1-1* lines were sampled at ZT4 instead of ZT3. DNA was purified using the PCR Clean-Up Kit (Qiagen) instead of by phenol-chloroform extraction. Immunoprecipitated DNA was analyzed with Applied Biosystems FAST real-time PCR in FAST mode (using presets). For each biological replicate immunoprecipitated DNA was normalized to the input DNA as in ref. 31, and each of these values was expressed relative to the *cca1-1* line set to a value of 1. A one-tailed paired *t* test was performed to assess the statistical significance of enrichment in the CCA1p:CCA1-GFP line compared with *cca1-1* plants for each primer pair used across biological replicates. Primer pairs used in ChIP experiments are listed in Table S1.

**Freezing-Tolerance Tests.** Electrolyte leakage assays were performed as described in ref. 16. For cold acclimation, plants were transferred to 4 °C at ZT4 for 7 d under a 12-h photoperiod. Assays for acclimated and nonacclimated plants started at ~ZT2 in all biological replicates.

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