

Loss of the circadian clock-associated protein 1 in *Arabidopsis* results in altered clock-regulated gene expression

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ABSTRACT Little is known about plant circadian oscillators, in spite of how important they are to sessile plants, which require accurate timekeepers that enable the plants to respond to their environment. Previously, we identified a circadian clock-associated (*CCA1*) gene that encodes an Myb-related protein that is associated with phytochrome control and circadian regulation in plants. To understand the role *CCA1* plays in phytochrome and circadian regulation, we have isolated an *Arabidopsis* line with a T DNA insertion that results in the loss of *CCA1* RNA, of *CCA1* protein, and of an *Lhcb*-promoter binding activity. This mutation affects the circadian expression of all four clock-controlled genes that we examined. The results show that, despite their similarity, *CCA1* and *LHY* are only partially redundant. The lack of *CCA1* also affects the phytochrome regulation of gene expression, suggesting that *CCA1* has an additional role in a signal transduction pathway from light, possibly acting at the point of integration between phytochrome and the clock. Our results indicate that *CCA1* is an important clock-associated protein involved in circadian regulation of gene expression.

Organisms have internal clocks to regulate physiological and cellular processes. The timekeepers, or oscillators, controlling these clocks are proposed to be negative autoregulatory feedback loops in which one or more gene products feed back and repress their own expression (1). The oscillator can be reset by input pathways from environmental cues, such as light and temperature, and, in turn, can regulate overt rhythmicity. Although much has been learned about circadian clocks in other organisms, little is known about the molecular basis of plant circadian oscillators. Plant homologs have not been identified yet for any of the known circadian genes from other organisms, including *frq* from *Neurospora*, *per* and *tim* from *Drosophila*, *mper* from mice, and the *kai* genes from the cyanobacterium *Synechococcus* (1, 2). An *Arabidopsis* mutant, *toc1*, that affects the period of clock-controlled processes has been isolated; however, the corresponding gene has not been cloned, and its role in the oscillator is not known (3).

Recently, two *Arabidopsis* Myb-related genes, *CCA1* (circadian clock-associated) and *LHY*, have been shown to have an important function in the circadian control of a number of plant processes (4, 5). *CCA1* and *LHY* are 60% similar at the amino acid level, with regions of identity extending throughout the protein. Constitutive expression of either gene affects multiple processes controlled by circadian rhythms, including oscillations of circadian clock-controlled genes (4, 5). Furthermore, both *CCA1* and *LHY* RNAs showed circadian oscillations after a period of entrainment and showed feedback inhibition of their own synthesis. Constitutive expression of *CCA1* represses the expression of the *LHY* gene (4). *CCA1* also is regulated by the plant photoreceptor phytochrome (4).

To understand how *CCA1* might function in the circadian control of plant processes and in the signal transduction pathway from phytochrome, we have identified a null mutation that results in the loss of *CCA1*. We show here that loss of *CCA1* affects the expression of all the clock-controlled genes examined, as well as the phytochrome regulation of gene expression. These data show that *CCA1* plays a central role in both phytochrome and circadian regulation of gene expression.

MATERIALS AND METHODS

Plant Materials and Growth. Light-grown plants were grown on MS2S medium [Murashige and Skoog salts (GIBCO/BRL), 0.05% Mes (pH 5.7), 0.8% Phytagar (GIBCO/BRL), and 2% sucrose] or on soil (both methods gave the same results) under light:dark cycles [12 h of light ($125 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$):12 h of darkness; $E = 1 \text{ mol of photons}$] at 23°C for 17 days before harvesting at dawn. For circadian experiments, plants were transferred to constant light ($100 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$) at 23°C. Etiolated plants were grown for 6 days in the dark before being given 1 min of red light and then being harvested at indicated intervals.

T DNA Insertion-Line Identification. DNA prepared from T DNA insertion lines in a Wassilewskija background (*Arabidopsis* Biological Resource Center stock CS6502; seeds were donated by K. Feldmann, University of Arizona, Tucson, AZ; pools of DNA were prepared by C. Lin, University of California, Los Angeles) was screened by PCR by using T DNA left and right border-specific primers (6) and primers specific for *CCA1* 5'-TGAGATTCTCCATTCCCGTAGCTTCTGG and 3'-ATCCGTTTGGGATCTTTCTGTTCCACATG. One pool was found to give a PCR product with the left T DNA border primer in conjunction with either the 3' or the 5' *CCA1* primers. Southern blot analysis confirmed that this product included regions of the *CCA1* gene. Progressively smaller pools of DNA containing the T DNA insertion in *CCA1* were identified by PCR until a single line with a T DNA insert in the *CCA1* gene was isolated.

RNA and Protein Analyses. RNA and protein analyses were carried out as described (4, 7, 8). A DNA template for synthesis of an RNA probe for *CAT2* was synthesized by PCR amplification of a genomic DNA fragment containing nucleotides 1,553–1,773 (9) with primers *CAT2S* (5'-AACGCGTGAAA-GAATTCTTGATTGGCC-3') and *T7CAT2A* (5'-**TGTAAT-ACGACTCACTATAGGGGACCTTTCATCAAGTAACAC-C-3'**, with the T7 promoter region in bold).

Electrophoretic Mobility-Shift Assays. Protein extracts were prepared from 3-week-old soil-grown plants as described (7), except that phosphatase inhibitors (50 mM NaF, 0.2 mM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, 5 mM NH_4VO_3 , and 1 mM EGTA) were added to all the solutions, and the whole-cell extracts were desalted on a Sephadex G-25 column (Amersham Pharmacia). The proteins were then preincubated at 30°C for 30 min with 50 mM Tris·HCl (pH 7.5), 0.1 mM Na_2EDTA , 5 mM DTT, and

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2 mM MnCl₂, and electrophoretic mobility-shift assays were carried out as described (7). The wild-type promoter fragment designated A2 (7) was used unless otherwise stated.

RESULTS

Isolation of a *CCA1*-Null Line. We used a PCR-based procedure (6) to isolate a *CCA1*-null line (*cca1-1*) of *Arabidopsis*, in which the *CCA1* gene was interrupted by a T DNA insertion. Fig. 1A shows a diagram of the large T DNA insertion that disrupts intron 4 of the *CCA1* gene. Partial sequencing of the insertion site showed that the T DNA insert was in the fourth intron of the *CCA1* gene. There is a small (24-bp) deletion in the *CCA1* sequence and another small (26-bp) fragment of non-*CCA1* or T DNA at the insertion site. Both ends of the T DNA insert have left borders, suggesting

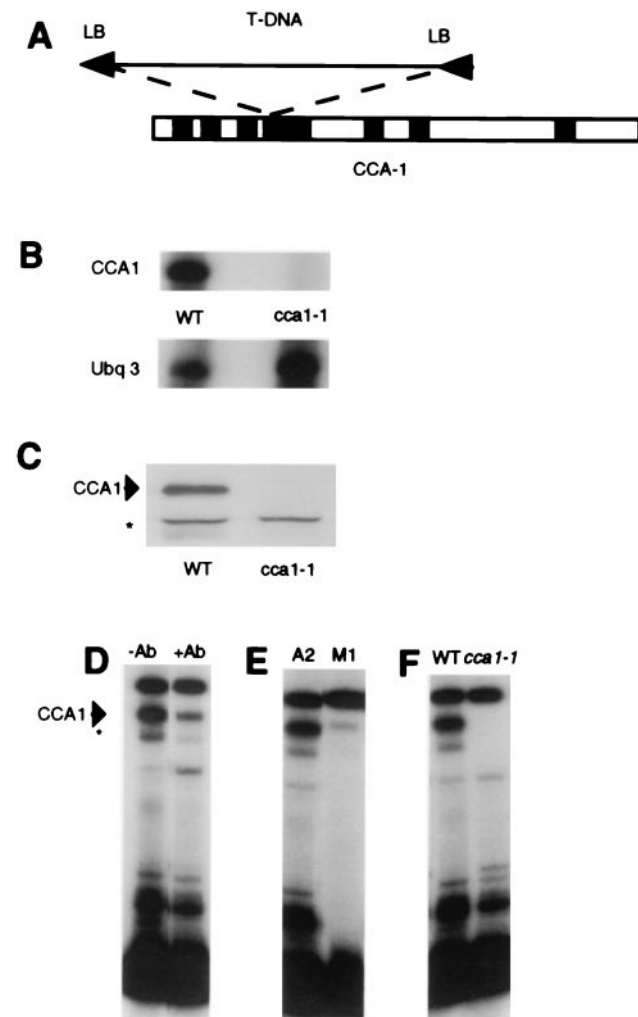


FIG. 1. Identification of the *CCA1*-null line. (A) T DNA insertion site in *CCA1*. Black boxes, introns; white boxes, coding regions; LB, left border. (B) The *cca1-1* line lacks *CCA1* RNA. RNA from wild-type (WT) Wassilewskija ecotype and *cca1-1* plants was used as a template for RNase protection analysis with probes for *CCA1* and a control, *UBQ3*. (C) *CCA1* protein is absent in the *cca1-1* plants. Western blots of wild-type (WT) and proteins from *cca1-1* plants were assayed with *CCA1* antibodies. The upper band (arrowhead) is *CCA1*; the lower band (asterisk) is a nonspecific band routinely seen in Western blot analyses carried out with *CCA1* antibodies. (D-F) Electrophoretic mobility-shift assays showing a major (arrowhead) and a minor (asterisk) *CCA1*-binding activity; these activities are reduced after preincubation of protein extracts with *CCA1* Abs and when a mutated promoter fragment (M1) is used in the binding assay, and the activities are missing completely in protein extracts from *cca1-1* plants.

that two of the 17-kb T DNAs have been inserted in a head-to-head configuration, resulting in a 34-kb insertion in the *CCA1* gene.

To examine whether the insertion was sufficient to disrupt correct transcription and translation, we determined RNA and protein levels in the *cca1-1* plants. Fig. 1B shows the results of an RNase protection analysis and shows that this line has no detectable *CCA1* RNA. Because the T DNA insertion is located in the fourth intron of the *CCA1* gene, we considered the possibility that a truncated RNA might be produced. Therefore, Northern analysis of the *CCA1* RNA also was performed by using an RNA probe synthesized from a template made to the -68 to +186 end of the gene (7). The results of this experiment confirmed that there were no detectable *CCA1* transcripts from the 5' end of the *CCA1* gene (data not shown). Furthermore, the *cca1-1* line had no detectable *CCA1* protein (Fig. 1C).

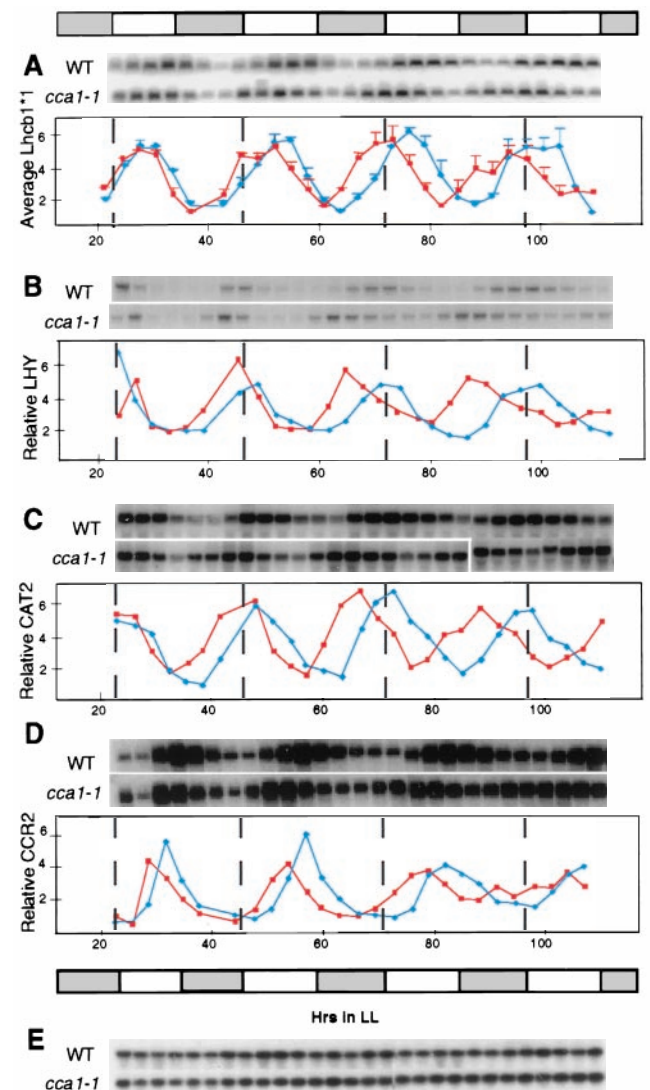


FIG. 2. Circadian oscillations of clock-controlled genes in the *cca1-1* line. The expression of *Lhcb1*1* (A), *LHY* (B), *CAT2* (C), *CCR2* (D), and *UBQ10* (E) genes. Each experiment was repeated at least three times with identical results. One representative autoradiogram is shown for *Lhcb1*1*, *LHY*, *CAT2*, and *CCR2*. Quantitation based on expression of the *UBQ10* control for the Northern blots for *LHY*, *CAT2*, and *CCR2* is shown. Because quantitation of individual experiments with *Lhcb1*1* showed some smaller secondary peaks, the results of the three experiments were averaged, and are shown \pm SEM. Red lines, *cca1-1*; blue lines, wild-type (WT) Wassilewskija ecotype. The bar represents the subjective light conditions.

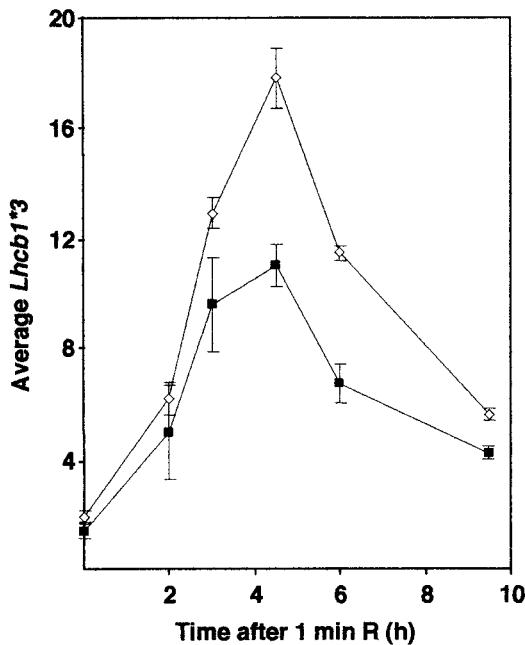


FIG. 3. Phytochrome induction in the *cca1-1* line. Northern analysis of *Lhcb1*3* RNA in 6-day-old etiolated plants after a 1-min exposure to red light. Values were normalized to a *UBQ3* control. Open diamonds, wild-type (WT) Wassilewskija ecotype; closed squares, *cca1-1*. The results from three independent experiments were averaged and are shown \pm SEM.

CCA1 was isolated originally as a factor that can bind well characterized regions of circadian- and phytochrome-regulated *Lhcb* (also known as *CAB*) promoters (7, 10–12). Analysis of the binding activity of proteins isolated from wild-type plants to an *Lhcb1*3* promoter probe showed that two DNA–protein complexes were much reduced when they were preincubated with an anti-CCA1 antibody (Fig. 1D). Fig. 1E shows that these complexes were also reduced when the wild-type DNA probe A2 (7) was replaced by M1, which has mutations in the sequences that have been shown to be important for recombinant CCA1 binding (7). These results indicate that CCA1 is a component of these complexes. We, therefore, examined the binding activity of plant extracts isolated from *cca1-1* plants. Fig. 1F shows that the *Lhcb1*3*-promoter binding complexes were undetectable in protein extracts isolated from *cca1-1* plants. Taken together, these results confirm that *cca1-1* is a null line lacking CCA1 RNA, CCA1 protein, and an *Lhcb1*3*-promoter binding activity.

Circadian Control of Gene Expression Is Altered in *cca1-1* Plants. We next tested whether the circadian regulation of gene expression was altered in the *cca1-1* line. *cca1-1* plants were transferred to constant light after entrainment in light:dark conditions (12 h of light:12 h of darkness). Fig. 2A shows that the period of *Lhcb1*1* circadian oscillations was \approx 3 h shorter (the average of the distances between the peaks measured for three independent experiments, carried out over four cycles) than that in wild-type plants grown under the same conditions. On the first day of the experiment, the timing of the increase of *Lhcb1*1* RNA in *cca1-1* plants was almost identical to that in wild-type plants. However, by the fourth day of the experiment, the *Lhcb1*1* RNA peaked much earlier in *cca1-1* than in wild-type plants.

Thus, loss of CCA1 affects the period of *Lhcb1*1* gene expression. However, oscillations were not lost completely. We have shown that CCA1 binds directly to *Lhcb* promoters (ref. 7; Fig. 1D), and we have found that LHY, a protein that is highly homologous to CCA1 (4, 5), also binds the *Lhcb* promoter (C. Andronis, S. Sugano, and E.M.T., unpublished

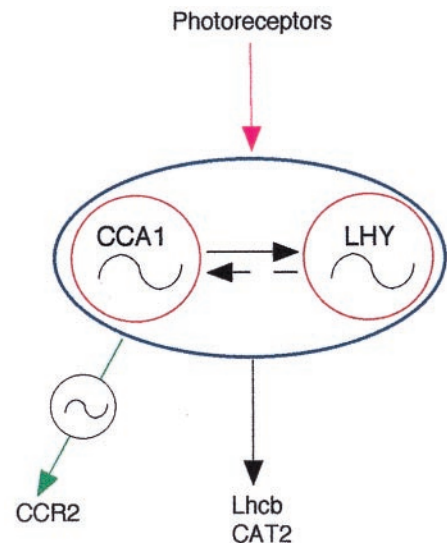


FIG. 4. Diagram of a possible role of CCA1 in circadian rhythms. The solid and dashed arrows represent potential interactions between CCA1 and LHY. Constitutive expression of CCA1 represses the expression of the *LHY* gene (4); the effect of constitutive expression of LHY on CCA1 has not yet been established. The wavy lines represent potential oscillators, and the solid line around CCA1 and LHY indicates possible redundant functioning of CCA1 and LHY in regulating rhythms of target genes.

data). Therefore, LHY may be responsible for the control of *Lhcb1*1* expression in the absence of CCA1. Fig. 2B shows that the period of LHY oscillations was indeed also \approx 3 h shorter in the *cca1-1* plants, resembling the period of *Lhcb1*1* oscillations. By the fourth day in continuous light, the peak of LHY expression in *cca1-1* plants occurred approximately 12 h earlier than it did in the wild-type plants. These results show not only that there is a strong correlation between the period of oscillations of LHY and that of *Lhcb1*1* but also that the absence of CCA1 affects the period of the LHY RNA rhythm. In contrast, the *elf3* mutation, which has been postulated to affect the input pathway to the circadian oscillator (13, 14), causes a loss of LHY RNA oscillations in constant-light conditions (5).

If both LHY and CCA1 are associated closely with a central oscillator in *Arabidopsis* cells, then a shortened period of oscillations also would be expected in the expression of other clock-controlled genes. Indeed, *CAT2* RNA (catalase 2; ref. 15), which peaks around subjective dawn in wild-type plants, showed a shorter period of oscillation in *cca1-1* plants than in wild-type plants (Fig. 2C). *CCR2* (also known as *AtGRP7*), which encodes an RNA binding protein, is a clock-controlled gene whose RNA peaks considerably later in the cycle than the RNA of LHY, *CAT2*, and *Lhcb1*1* (16, 17). Fig. 2D shows that circadian oscillations of *CCR2* clearly were affected in the *cca1-1* plants. However, it is difficult to determine conclusively whether this alteration is a phase or period shift.

The *cca1-1* Line Shows a Reduced Phytochrome Induction of *Lhcb1*3* Expression. Environmental light perception in plants is mediated by several families of photoreceptors, including the phytochromes and cryptochromes. Photoreceptors, in turn, entrain one or more circadian oscillators (18) but are also able to induce gene expression directly. In addition to binding to a region of the *Lhcb1*3* promoter required for phytochrome and circadian regulation, CCA1 has itself been shown to be induced transiently by the plant photoreceptor phytochrome (4). Thus, it also may play a role in the photo-transduction pathway from phytochrome. Indeed, the phytochrome induction of *Lhcb1*3* gene expression was reduced in *cca1-1* plants (Fig. 3), reaching approximately 60% (average of

three experiments) of the induction in the wild-type plants. Recently, it was reported (19) that imbibition of seeds is sufficient to start some circadian rhythms in *Arabidopsis* seedlings, and the timing of the light treatment might be expected to affect the magnitude of *Lhcb1*3* induction observed. In our experiments, the times between imbibition and the red-light treatment given to the seedlings varied, but the amplitude of the induction of the *Lhcb* RNA did not. Our observations of the phytochrome induction of *Lhcb1*3* RNA in the *cca1-1* line are consistent with results obtained from plants transformed with a *CCA1* antisense construct (7). They suggest that *CCA1* may have a role not only in the circadian regulation of gene expression but also directly in a phototransduction pathway from phytochrome.

DISCUSSION

We have identified a null mutation that results in the absence of *CCA1*, a potential component of the circadian regulatory pathway in plants. Circadian regulation of all the clock-controlled genes we examined was found to be affected in these lines. Our results clearly show that *CCA1* is playing a central role in circadian control of gene expression in plants. The fact that circadian oscillations are not lost in the *cca1-1* line suggests that many features of circadian control are still functional. It is possible that there is another protein partially able to compensate for the loss of *CCA1*. An obvious candidate is *LHY*, which has a very high degree of homology with *CCA1* and strong similarities in the phenotypes of the overexpressing lines (4, 5). Furthermore, we have shown (Fig. 2A and B) that there is a strong correlation between the period of oscillations of *LHY* and *Lhcb1*1*. However, the functions of *CCA1* and *LHY* are not completely redundant, because the loss of *CCA1* results in a clearly altered circadian gene expression phenotype. It remains to be seen whether *LHY* compensates for the loss of *CCA1* in the *cca1-1* plants, and it would be of interest to examine the effects of a *CCA1-LHY* double loss-of-function mutation on circadian rhythms.

The period of oscillations of *LHY*, *CAT2*, and *Lhcb1*1* were clearly shorter in the *cca1-1* line. However, the effect of the null mutation on *CCR2* was less clear but may have involved a phase shift. If *CCR2* is phase-shifted in the *cca1-1* line, it would indicate that neither *CCA1* nor *LHY* is controlling *CCR2* oscillations directly and would suggest that there is at least one additional oscillator in the cell. The existence of multiple oscillators in plants has been suggested by physiological studies in bean and by an analysis of gene expression in very young tobacco seedlings (20–22). Alternatively, the pattern of *CCR2* oscillations that we observed might be a result of the autoregulatory *CCR2* oscillator winding down (16). However, it is also possible that, like the other clock-controlled genes we examined, *CCR2* has an altered period in *cca1-1* plants.

In addition, we have shown that *CCA1* is involved directly in light regulation of gene expression, acting in the signal transduction pathway from phytochrome to *Lhcb1*3*. In this respect, *CCA1* resembles the *white collar* genes of *Neurospora* whose products are involved in light-regulated transcriptional activation of gene expression and also play a role in the *Neurospora* circadian oscillator (23). These results are consistent with recent findings that circadian oscillations of a reporter gene driven by an *Lhcb* promoter are altered in phytochrome A and B mutants in *Arabidopsis* (24). It is possible that *CCA1* provides a molecular link between phytochrome and the circadian oscillator in plant cells.

Although further experiments will be needed to elaborate fully the role of *CCA1* and other proteins in circadian regu-

lation, it is clear from the data presented here and from our studies on an overexpressing line (4) that *CCA1* plays a significant role. We cannot eliminate the possibility that *CCA1* may be part of an output from the oscillator that loops back to the input to the clock (25, 26) or acts directly as an input. However, our evidence is consistent with the idea that *CCA1* may act as part of a central oscillator as illustrated in Fig. 4. In this model, a possible explanation for the shorter period of gene expression seen in the *cca1-1* plants would be that, in wild-type plants, *LHY* and *CCA1* may form heterodimers; however, in the absence of *CCA1*, *LHY* may form homodimers that can maintain oscillations and affect the feedback inhibition differently.

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