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Constitutive Expression of the *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) Gene Disrupts Circadian Rhythms and Suppresses Its Own Expression

Zhi-Yong Wang† and Elaine M. Tobin*
Department of Molecular, Cell, and Developmental Biology
UCLA
Los Angeles, California 90095-1606

Summary

The *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) gene encodes a MYB-related transcription factor involved in the phytochrome induction of a light-harvesting chlorophyll *a*/*b*-protein (*Lhcb)* gene. Expression of the *CCA1* gene is transiently induced by phytochrome and oscillates with a circadian rhythm. Constitutive expression of CCA1 protein in transgenic plants abolished the circadian rhythm of several genes with dramatically different phases. These plants also had longer hypocotyls and delayed flowering, developmental processes regulated by light and the circadian clock. Furthermore, the expression of both endogenous *CCA1* and the related *LHY* gene was suppressed. Our results suggest that CCA1 is a part of a feedback loop that is closely associated with the circadian clock in *Arabidopsis.*

Introduction

Photoreceptors and circadian clocks are universal mechanisms for sensing and responding to the light environment. The phase of circadian rhythms can be set by light and dark transitions, but these rhythms can persist even in the absence of such external time cues. In addition to regulating daily activities, photoreceptors and circadian clocks are also involved in the seasonal regulation of processes such as flowering, a developmental response to day length called photoperiodism (Thomas and Vince-Prue, 1997). However, although the importance of circadian rhythms in plant growth and development has long been recognized, and *Arabidopsis* mutants with altered clock function have been isolated (Millar et al., 1995a), no clock component has yet been identified in plants.

Studies of the clock mechanisms of *Neurospora* and *Drosophila* have demonstrated that in these organisms the central oscillating mechanisms involve a feedback loop whereby transcription is inhibited as a result of the accumulation of the encoded proteins (Dunlap, 1996). Clock genes isolated in both organisms, *FRQ* and *WC-2* of *Neurospora* and *PER* and *TIM* of *Drosophila*, satisfy a set of basic criteria proposed for components of the circadian oscillator (Aronson et al., 1994; Kay and Millar, 1995; Dunlap, 1996; Crosthwaite et al., 1997). These genes encode nuclear proteins that are predicted to function by regulating transcription (Dunlap, 1996). However, only the WC-2 protein has been shown to bind to DNA (Crosthwaite et al., 1997; Linden and Macino, 1997). The demonstration that *WC-2* is a component of the circadian oscillator also supports a central role of the circadian clock in light responses of *Neurospora*, since the *wc-2* mutant is defective in almost all photoresponses (Crosthwaite et al., 1997). Recently, putative clock component genes have also been identified in mammals (King et al., 1997; Sun et al., 1997; Tei et al., 1997).

There is a wide range of processes in plants that show a circadian rhythm. These include movement of organs such as leaves and petals, stomata opening, stem elongation, sensitivity to light of floral induction, metabolic processes such as respiration and photosynthesis, and expression of a large number of different genes (Bünning, 1936; and reviewed by Piechulla, 1993; McClung and Kay, 1994; Anderson and Kay, 1996; Kreps and Kay, 1997). It is believed that the circadian rhythm of gene expression is part of the underlying mechanism for many, if not all, of the rhythms in metabolic and developmental processes.

Most of the circadian-regulated genes studied previously are related to photosynthesis and have a peak level of expression in the morning. A few exceptions include the *cold, circadian rhythm* RNA binding protein 2 (*CCR2/AtGRP7*) gene (Carpenter et al., 1994; Heintzen et al., 1997) and the Catalase3 (*CAT3*) gene (Zhong and McClung, 1996), which peak in the evening. The rhythmic expression of these genes is thought to reflect outputs from the circadian oscillator. The CCR2/AtGRP7 protein has been shown to be involved in the oscillation of its own transcript and the transcript of a related gene. However, it is not involved in the circadian rhythms of other genes and is therefore believed to be a slave oscillator that is part of an output pathway of the master clock (Heintzen et al., 1997).

There is an intimate relationship between certain photoreceptors and the circadian clock, including the entrainment and resetting of the clock by light, the "gating" of photoreceptor activity by the clock, and the concerted action of photoreceptors and circadian clocks on common cellular and physiological processes (Johnson, 1994; Millar and Kay, 1997). In plants, phototransduction not only sets the phase, but also affects the amplitude and period of circadian rhythms (Millar et al., 1995b). Members of the phytochrome family of plant photoreceptors, which can exist in two photochemically interconvertible forms and are involved in regulation of plant development and growth (Quail, 1997), play important roles in regulating clock activities (Millar and Kay, 1997; Thomas and Vince-Prue, 1997). Many of the genes regulated by phytochrome are also regulated by the circadian clock (Piechulla, 1993).

The *Lhcb* gene family (previously designated *CAB*; Jansson et al., 1992), which encodes apoproteins of the light-harvesting complex associated with photosystem II, has been one of the model systems for studies of both phytochrome and circadian regulation of gene expression. These studies have led to the identification of

*To whom correspondence should be addressed.
†Present address: Plant Biology Laboratory, Salk Institute, San Diego, California 92186.

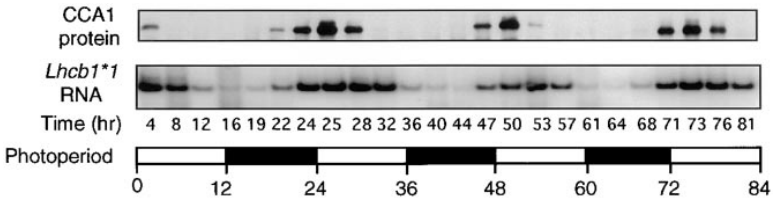

Figure 1. *CCA1* Expression Oscillates with a Diurnal Rhythm

Arabidopsis plants were grown and maintained on soil in LD 12:12 for 3 weeks, then seedlings were collected about every 4 hr. CCA1 protein was detected with affinity-purified anti-CCA1 antibodies after blotting. The *Lhcb1*1* RNA was detected by quantitative RT-PCR. Open and closed bars represent light and dark photoperiods, respectively. Time 0, light-on of the day the first sample was taken.

promoter elements involved in phytochrome induction as well as promoter regions important for the circadian rhythm of gene expression (Anderson and Kay, 1995; Carré and Kay, 1995; Kenigsbuch and Tobin, 1995). Several protein factors interacting with some of these promoter regions have also been identified; however, in vivo function has only been demonstrated for one of these factors (Wang et al., 1997). These studies have also demonstrated the intimate link between phytochrome regulation and the circadian clock in regulating transcription (Nagy et al., 1993; Millar and Kay, 1996; Anderson et al., 1997).

We have previously isolated and characterized a transcription factor, designated CCA1, which binds to a region of an *Arabidopsis Lhcb* promoter (*Lhcb1*3*) that is necessary for its phytochrome responsiveness (Wang et al., 1997). CCA1 is a Myb-related protein that binds to at least two of the *Lhcb* genes of *Arabidopsis*, *Lhcb1*1* and *Lhcb1*3*, at a sequence that is conserved in *Lhcb* genes of many species. *CCA1* RNA showed a transient increase when etiolated seedlings were transferred to white light, and this increase preceded the increase in *Lhcb* RNA. Most importantly, expression of antisense *CCA1* RNA in transgenic plants reduced the phytochrome induction of the *Lhcb1*3* gene, demonstrating that CCA1 can act as a transcriptional activator of this gene in vivo. It has also been shown that a 36 bp region of the closely related *Lhcb1*1* promoter that is sufficient to confer a circadian rhythm of expression to a reporter gene (Carré and Kay, 1995) is bound by CCA1 (Wang et al., 1997).

We have now further investigated the role of *CCA1* and have found that it plays a central role in circadian regulation. It has satisfied many of the criteria expected for a component of the circadian oscillator, including rhythmic expression, disruption of multiple circadian rhythms by its constitutive expression, being subject to negative feedback regulation, and being induced by light signals. In the accompanying paper, the discovery and characterization of a closely related gene, *LHY*, is described. Overexpression of *LHY* caused similar long hypocotyl and late flowering phenotypes as the overexpression of *CCA1* (Schaffer et al., 1998, this issue of *Cell*). Together our findings demonstrate that *CCA1* and *LHY* are closely associated with the circadian clock in *Arabidopsis*. They also provide a possible molecular link for light and circadian clock regulation of gene expression and plant development.

Results

Expression of the *CCA1* Gene Shows a Diurnal Rhythm

We measured the levels of CCA1 protein during growth of seedlings in a 12 hr light and 12 hr dark photoperiod (LD 12:12). Figure 1 shows that CCA1 protein was expressed rhythmically, with peak levels occurring around 1 hr after dawn. The diurnal rhythm of the expression of the *Lhcb1*1* gene is also shown. The peaks for the CCA1 protein occur slightly earlier than those of the *Lhcb* RNA. These results are consistent with our previous observation that CCA1 is an activator of *Lhcb* transcription and is of primary importance in the regulated expression of the *Lhcb* genes. They also suggested that the expression of *CCA1* might be regulated by a circadian rhythm and be responsible for the circadian rhythm of the expression of *Lhcb* genes.

Constitutive Expression of CCA1 Results in Longer Hypocotyls and Substantially Delayed Flowering

In order to investigate the functions of CCA1, we transformed *Arabidopsis* plants with a fusion construct that placed the coding region of the *CCA1* cDNA under the control of the CaMV 35S promoter, which directs constitutive high-level expression in plants. From 53 primary transformants, we obtained homozygous seeds of 33 transformed lines that each had a single site of insertion for further analysis. These lines (CCA1-ox plants) displayed a range of alterations in hypocotyl length and in flowering time. Figure 2A shows the range of hypocotyl lengths in wild types and a sample of CCA1-ox transgenic lines for five-day-old seedlings grown in LD 16:8 photoperiods. In 14 of the 17 transgenic lines tested, the hypocotyl length was significantly longer than wild type. Figure 2B shows the time to bolting for 23 of the CCA1-ox transgenic lines, representing the range seen in all 33 lines. Many of the transgenic lines showed a substantial delay in flowering. In the most extreme case, flowering was delayed until 56 days, while the wild-type plants bolted to flower in about 22–24 days. The number of rosette leaves at the time of bolting is also shown in Figure 2B. The correlation of the number of leaves with the bolting time in both wild-type and the transgenic plants indicates that the delayed flowering was due to developmental regulation rather than a general growth defect.

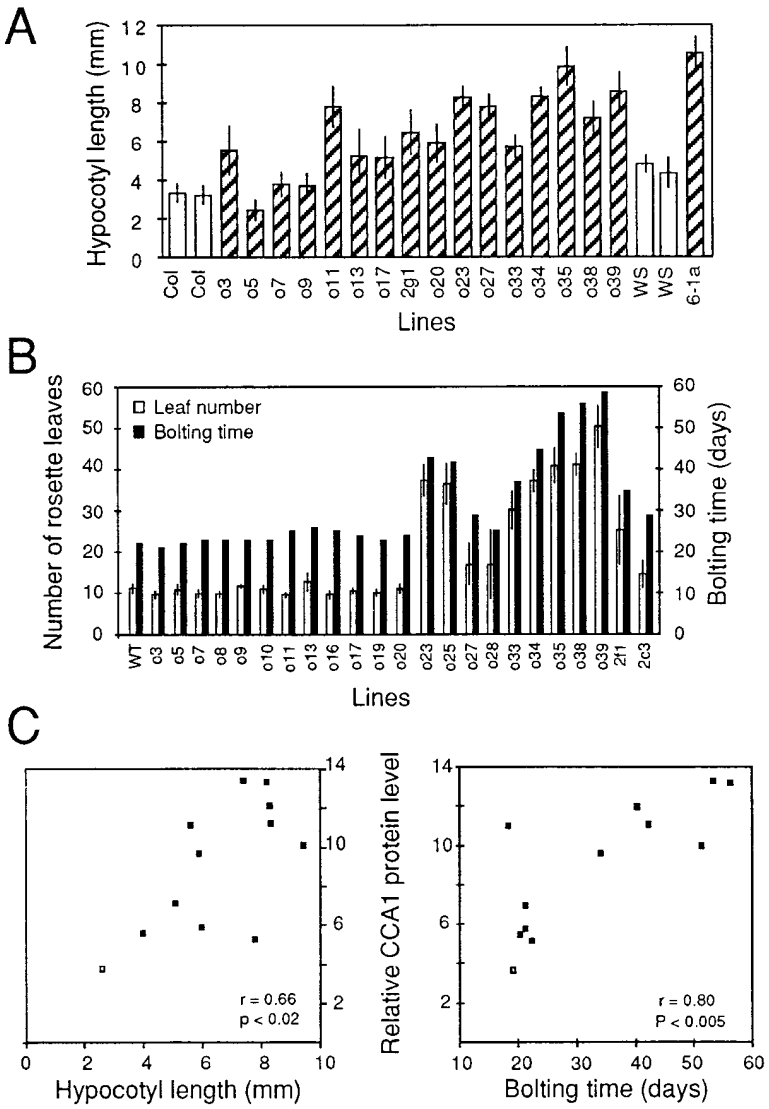

Figure 2. Constitutive Expression of CCA1 Protein Affects Hypocotyl Growth in Light and Causes Delayed Flowering

(A) CCA1-ox plants have longer hypocotyls than wild-type plants. Average hypocotyl lengths (± SD) of wild-type and different lines of CCA1-ox transgenic plants grown for 5 days in LD 16:8 light cycles. Col, wild type, Columbia ecotype; WS, wild type, WS ecotype; o3–o39, CCA1-ox lines in Col background; 6-1a, CCA1-ox in WS background.

(B) CCA1-ox transgenic plants have delayed flowering in long-day (LD 16:8) photoperiods. Mean time to bolting (closed bars) and the average number of rosette leaves (open bars ± SD) are shown for wild-type (WT) and CCA1-ox transgenic lines (3–2c3).

(C) The CCA1 protein level and the severity of the phenotypes are correlated in different transgenic lines. Scatter plots illustrating the correlation between the relative level of CCA1 protein and hypocotyl lengths (left) or bolting time (right) in a wild-type sample (open symbol) and eleven CCA1-ox lines (closed symbols). The relative levels of CCA1 protein in dark-grown seedlings were determined by Western blotting. The hypocotyl lengths and bolting times are as shown in Figures 2A and 2B. r, correlation coefficient. P, level of significance.

We demonstrated the dominant nature and cosegregation of the late flowering and longer hypocotyl phenotypes with the inserted transgene by analysis of the T2 generation of five randomly selected lines and of the F2 population of the back-crossing of plants homozygous for the insertion to wild-type plants. In all cases, cosegregation was observed (data not shown).

If the phenotypes were caused by the ectopic expression of the CCA1 protein, the severity of the phenotypes would be expected to correlate with the CCA1 protein quantity in the different transgenic lines. Figure 2C demonstrates that this is indeed the case for both the longer hypocotyl and delayed flowering phenotypes for 11 randomly selected independent transgenic lines. Since both hypocotyl elongation and flowering are developmental processes primarily regulated by light and the circadian clock, these observations suggested that CCA1 has important functions in light responses and circadian rhythms in addition to the expression of *Lhcb* genes.

CCA1 Shows a Circadian Rhythm of Expression

The observations of a diurnal rhythm of CCA1 expression and alteration of flowering time of the CCA1-ox transgenic plants led us to test whether CCA1 itself was regulated by the circadian clock and how its overexpression affected the circadian rhythm of *Lhcb* gene expression. Figure 3A shows that when wild-type plants were transferred from growth in LD 12:12 photoperiods into continuous light (LL), the levels of *CCA1* RNA and protein showed a circadian oscillation, both with peak levels occurring around subjective dawn. Also shown is the circadian rhythm of the *Lhcb1*1* RNA, which peaked about 4–8 hr after subjective dawn.

Constitutive Expression of CCA1 Abolishes the Circadian Rhythm of *Lhcb1*1* Gene Expression in Continuous Light (LL)

When the CCA1-ox plants were transferred to continuous light for an extended period, the circadian rhythm of the endogenous *Lhcb1*1* gene was absent. Figure

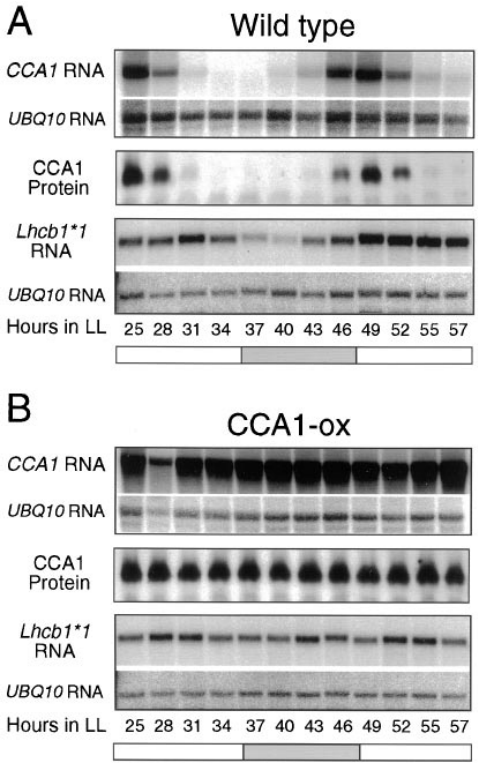

Figure 3. Constitutive Expression of CCA1 Protein Abolishes the Circadian Rhythm of *Lhcb1*1* RNA Expression in LL

CCA1 RNA, CCA1 protein, and *Lhcb1*1* RNA levels in wild-type and CCA1-ox (line o34) plants after shifting from LD 12:12 photoperiods into constant light. Plants were grown for 10 days on MS2S medium in LD 12:12 photoperiods, then shifted into constant light after light-on of day 11. After 25 hr, tissue was collected about every 3 hr for 32 hr. Two RNA gel blots were each hybridized with ^{32}P-labeled *CCA1* or *Lhcb1*1* RNA probe, and both with *UBQ10* RNA probe. Proteins from the same tissue were analyzed by Western blotting and detected with affinity-purified anti-CCA1 antibody. The open and shaded bars represent subjective light and dark photoperiods, respectively.

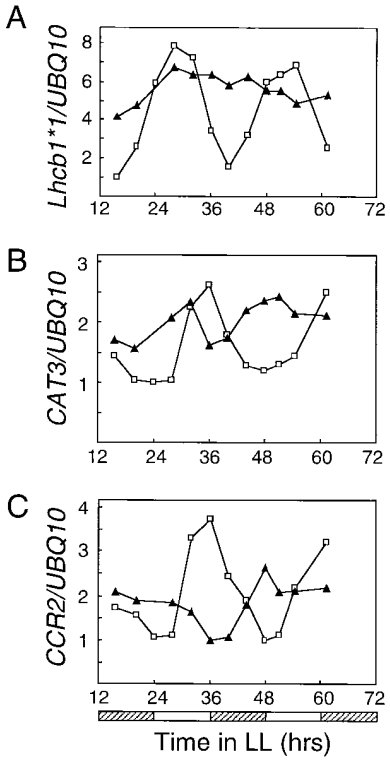

Figure 4. Constitutive Expression of CCA1 Disrupts Normal Circadian Rhythms of *Lhcb1*1*, *CAT3*, and *CCR2* Genes in LL Conditions

Expression of *Lhcb1*1* (A), *CAT3* (B), and *CCR2* (C) RNAs in wild-type and the CCA1-ox (line o38) plants grown for 10 days in LD 12:12 photoperiods, then shifted into continuous light (LL) after light-on of day 11 (time 0). Seedlings were harvested about every 4 hr. The *Lhcb1*1* and *CCR2* RNA levels were determined by quantitative RT-PCR and the *CAT3* RNA levels by RNA gel blotting. The *UBQ10* RNA levels were used as an internal control. Values were normalized to the lowest value of the wild-type samples. The bar containing open and hatched boxes shows the subjective light and dark photoperiods, respectively.

3B shows the expression of the *CCA1* RNA and protein and the *Lhcb1*1* RNA in CCA1-ox plants transferred from LD 12:12 cycles into LL. The *CCA1* RNA was expressed at a high level, and the CCA1 protein was expressed at a constant level similar to the peak level of wild-type plants. Interestingly, the normal circadian rhythm of the *Lhcb1*1* RNA was absent in these plants. The *Lhcb1*1* RNA was constantly expressed at a level similar to the peak level in wild-type plants. These results indicate that the circadian rhythm of the *Lhcb1*1* RNA is mediated by the oscillation of the CCA1 protein.

CCA1-ox Plants Also Show Altered Expression of Additional Circadian Regulated Genes in Continuous Light (LL)

We tested whether constitutive expression of CCA1 can affect the circadian rhythms of genes that are expressed differently from *Lhcb1*1*. In wild-type plants, the circadian rhythms of the *CCR2* and *CAT3* RNAs have phases that are nearly the opposite of the *Lhcb1*1* RNA. Furthermore, the *CAT3* RNA level damps to a high level in DD, while the *Lhcb* and *CCR2* RNAs damp to a low level (Carpenter et al., 1994; Zhong and McClung, 1996; Heintzen et al., 1997). A representative experiment comparing the expression pattern of these genes in LL to that of the *Lhcb1*1* gene in wild-type and CCA1-ox plants is shown in Figure 4. In the wild-type plants, the phase of *Lhcb1*1* RNA oscillation (Figure 4A) was nearly the opposite of that of the *CAT3* (Figure 4B) and *CCR2* (Figure 4C) RNAs, as previously observed. In the CCA1-ox plants, the rhythm of expression of the *Lhcb1*1* gene was virtually abolished. The expression patterns of *CCR2* and *CAT3* RNAs were also profoundly affected. Although there was some fluctuation in the *CCR2* and *CAT3* RNA levels in the CCA1-ox plants, the amplitudes of these fluctuations were reduced compared to wild-type plants and they did not show circadian periodicity. Similar results were obtained with a second CCA1-ox line (o34). Thus, constitutive expression of CCA1 can affect circadian rhythms with different phases in LL conditions.

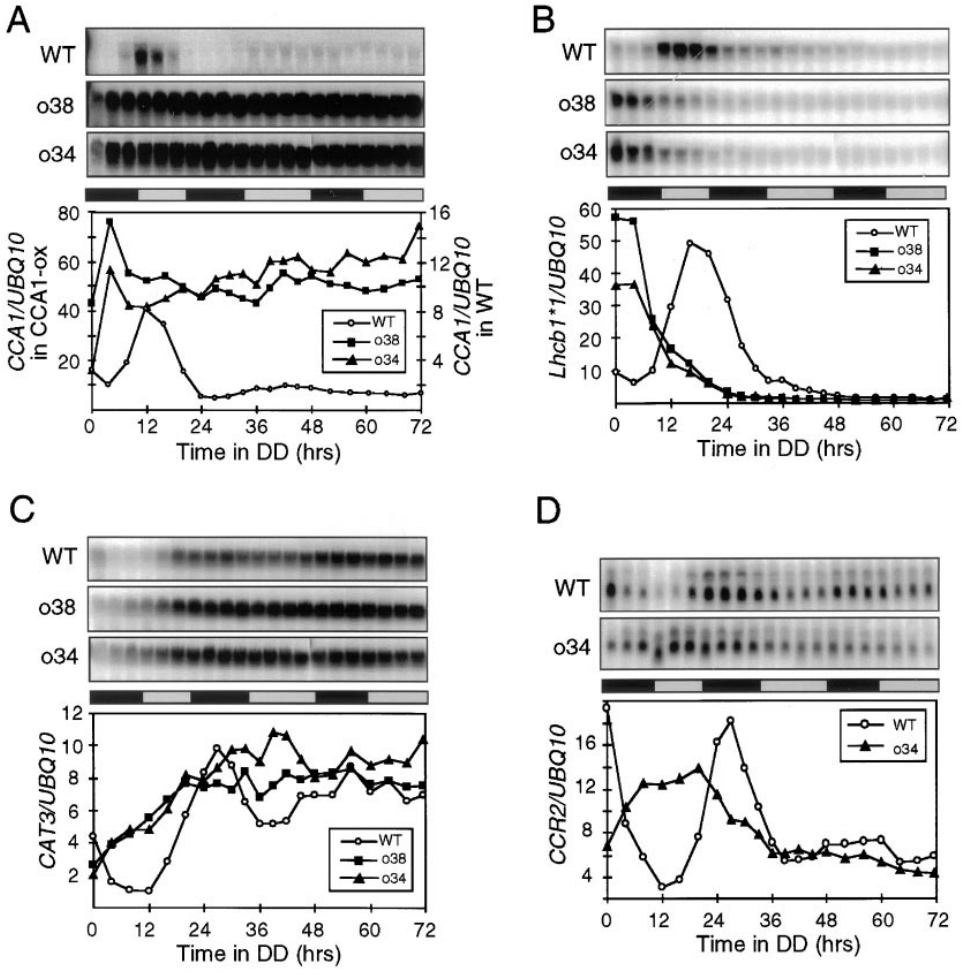

Figure 5. Constitutive Expression of CCA1 Abolishes the Circadian Oscillation of the *Lhcb1*1*, *CAT3*, and *CCR2* RNAs in DD

Quantitation of *CCA1*, *Lhcb1*1*, *CAT3*, and *CCR2* RNAs after plants grown in LD 12:12 photoperiods were shifted into DD. Wild-type and CCA1-ox (lines o34 and o38) plants were grown in constant light for 4 days, entrained in LD 12:12 cycles for 6 days, then shifted into constant darkness. Samples were taken about every 3–4 hr. RNA gel blot of total RNA (15 μg/lane) was hybridized with the probes of the *CCA1* (A), *Lhcb1*1* (B), *CAT3* (C), and *CCR2* (D) genes. Both autoradiographs and quantitation of the RNA gel blots are shown. The signals of *UBQ10* RNA (not shown) were used as the internal control for quantitation.

Constitutive Expression of CCA1 Causes Arrhythmic Expression of *Lhcb1*1*, *CAT3*, and *CCR2* RNA in Constant Darkness (DD)

Plant circadian rhythms often become undetectable after one to two cycles in conditions of prolonged darkness (DD). There is a mutant (*elf3*) that is thought to affect an input pathway to the clock and that has defective circadian rhythms in LL but not in DD (Hicks et al., 1996). It was therefore of interest to test whether constitutive expression of CCA1 affected circadian rhythms in DD conditions. Plants were entrained in LD 12:12 cycles and then transferred and maintained in complete darkness until tissue was harvested under a green safe light. Figure 5 shows the pattern of expression of four different genes that show rhythmic behavior in wild type and compares that rhythm to their expression in two lines of CCA1-ox plants.

In wild-type plants, the *CCA1* RNA level peaked about 12 hr after light-off, and then the oscillation damped to a low level of expression. In CCA1-ox plants, *CCA1* RNA level increased in the first 4 hr and then stayed at a level about 5-fold higher than the peak level of wild type (Figure 5A). The *Lhcb1*1* RNA level in wild-type plants peaked at about 16 hr after light-off as usually observed (Millar and Kay, 1996), though the second, low-amplitude peak was not obvious in this experiment (Figure 5B). In contrast, the *Lhcb1*1* RNA level decreased on transfer to DD and showed no oscillation in the CCA1-ox plants (Figure 5B).

The *CAT3* RNA level peaked first at about 27 hr after light-off and damped to a high level in wild-type plants (Figure 5C), similar to what was observed previously (Zhong et al., 1997). In CCA1-ox plants, the *CAT3* RNA level increased for the first 24 hr and was then maintained at a high level (Figure 5C). The circadian phase of the expression of *CCR2* gene was very similar to that of the *CAT3* gene in wild-type plants, but the *CCR2* RNA damped to a low level. In CCA1-ox plants, the *CCR2*

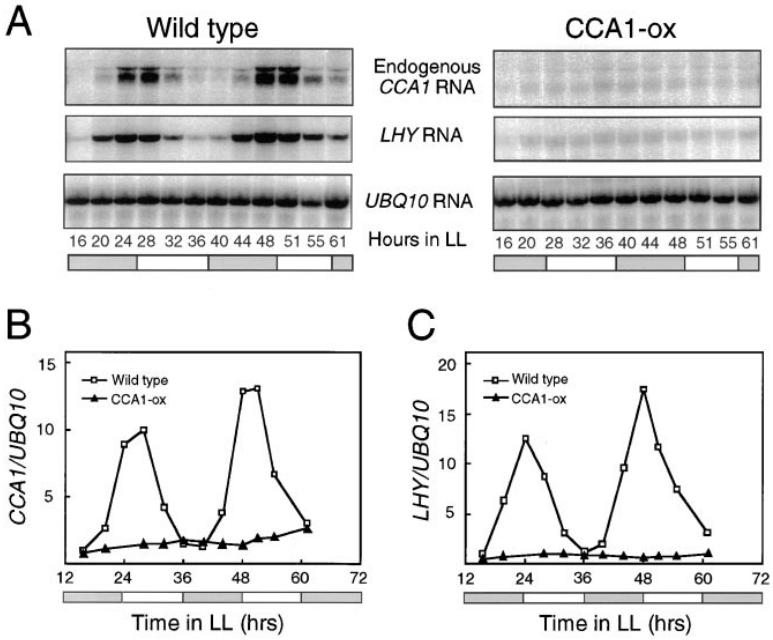

Figure 6. Expression of the *LHY* Gene and the Endogenous *CCA1* Gene Is Suppressed in the CCA1-ox Transgenic Plants

(A) Gel blot analysis of the RNA samples used in Figure 4. The blot was hybridized sequentially with the RNA probes containing the sequence of the *CCA1* 5′ untranslated region (UTR), which is not included in the CCA1-ox transgene, the *LHY* 5′UTR, and the *UBQ10* 3′UTR. Bars represent subjective photoperiods. Total RNA (15 μg) was loaded in each lane.

(B and C) Quantitation of the data shown in (A) normalized to the lowest value of wild-type samples.

RNA level increased in the first 16 hr in DD and then slowly dropped to a low level, showing no circadian oscillation (Figure 5D). These results demonstrate that the circadian rhythms of *Lhcb1*1*, *CAT3*, and *CCR2* gene expression are all disrupted in the CCA1-ox plants in DD.

The Rhythmic Expression of Both the Endogenous *CCA1* Gene and of the *LHY* Gene Is Repressed in CCA1-ox Plants

Our finding that the constitutive expression of CCA1 disrupted multiple circadian rhythms and at least two clock-regulated developmental processes, flowering time and hypocotyl elongation, suggested the possibility that *CCA1* might be a component of the circadian oscillator itself. Because in several other organisms the circadian clock comprises a negative auto regulatory feedback loop where the protein products of clock genes feedback suppress their own gene expression, we tested whether there might be a feedback regulatory mechanism governing the expression of the *CCA1* gene.

The expression of the endogenous *CCA1* gene was analyzed in the CCA1-ox plants grown in LD 12:12 cycles and transferred into continuous light. Figures 6A and 6B show that while the *CCA1* RNA level oscillated robustly in wild-type plants, the expression of the endogenous *CCA1* gene in the CCA1-ox plants remained at a constant low level that was equivalent to the trough level in wild-type plants. Similar results were obtained with a second CCA1-ox line (o34). Thus, a high level of CCA1 protein can cause nearly complete suppression of the *CCA1* gene in LL conditions.

The similarity of the phenotypes of plants constitutively expressing CCA1 to the gain-of-function *lhy* mutant plants described in the accompanying paper (Schaffer et al., 1998) led us to test whether CCA1 can also cause suppression of the *LHY* gene. Figures 6A and 6C demonstrate that the expression of *LHY* RNA, like that of *CCA1* RNA, was repressed in the CCA1-ox plants. We also note that the peak of *LHY* expression in wild-type plants was slightly earlier than that of *CCA1*. Thus, constitutive expression of CCA1 protein suppressed the expression and abolished the circadian rhythm of both *CCA1* and *LHY* genes, suggesting the possibility that these two genes are part of a feedback regulatory loop.

CCA1 Can Be Transiently Induced by Phytochrome Action

Another requirement expected for a circadian clock component is that the amount or activity should be affected by signals that reset the clock (Kay and Millar, 1995). In higher plants, the circadian clock can be entrained and reset by red and far-red light, which is perceived by phytochrome (Nagy et al., 1993). We tested whether such light treatments could affect the expression level of *CCA1*. Figure 7A shows that a brief red illumination of dark-grown plants was sufficient to induce a transient increase in *CCA1* RNA, peaking at about 1 hr, then declining by 8 hr to a level lower than before the light treatment. Figure 7B shows that the *CCA1* RNA could also be induced by red plus far-red light or far-red light alone, suggesting that the very low fluence response mediated by phytochrome A is responsible for this induction. These results demonstrate that *CCA1* RNA levels can be quickly induced by light signals that are known to reset the clock.

Discussion

The transcription factor CCA1 was originally isolated as a protein binding to an *Lhcb* promoter and involved in the phytochrome regulation of its expression. The results reported here now make it clear that CCA1 is also involved in the circadian rhythm of *Lhcb* gene expression. Furthermore, CCA1 plays an important role in circadian rhythms of expression of additional genes and in

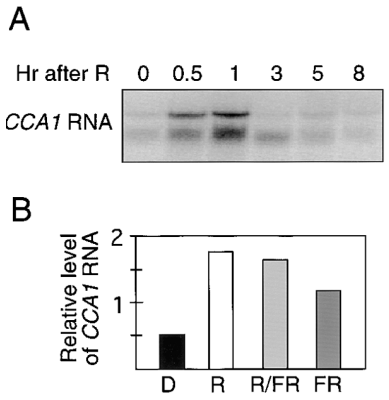

Figure 7. *CCA1* RNA Level Is Induced by Red and Far-Red Light in Etiolated Seedlings

(A) Time course of *CCA1* RNA induction after a 1.5 min R treatment of six-day-old etiolated seedlings. RNA gel blot of samples of total RNA (15 μg/lane) was hybridized with an RNA probe synthesized from *CCA1* cDNA clone 24.

(B) Phytochrome induction of *CCA1* RNA. Six-day-old etiolated seedlings were given no treatment (D), 30 s red light (R), 30 s red light followed by 10 min far-red light (R/FR), or 10 min far-red light (FR) treatment 1 hr before tissue was harvested. *CCA1* RNA was quantitated by competitive RT-PCR. The average of two experiments is shown. Similar results were obtained in a third experiment in which RNA was analyzed by RNA gel blotting.

developmental processes regulated by the circadian clock. The fact that the CCA1 protein level in the CCA1-ox plants was similar to the peak level in wild-type plants supports the idea that the phenotypes of the CCA1-ox plants were due to abolishing its rhythmic expression rather than due to a massive increase over the normal peak level of the CCA1 protein. Along with these findings, the demonstration that CCA1 acts as part of a negative feedback loop regulating its own expression raises the possibility that CCA1 is a part of the clock mechanism itself or is closely associated with it. Because CCA1 is involved in both phytochrome and circadian regulation of gene expression, it may also provide a molecular link for an understanding of the intimate relationship between photoreceptors and the circadian clock.

Function of *CCA1* in the Phytochrome Induction and the Circadian Rhythm of *Lhcb* Gene Expression

We have previously shown that CCA1 binds to a conserved promoter element of the *Lhcb1*3* gene and activates its expression in response to phytochrome induction. Expression of antisense *CCA1* RNA reduced the phytochrome induction of *Lhcb1*3* RNA, and the expression of *CCA1* itself was induced by light prior to the induction of *Lhcb1*3* RNA (Wang et al., 1997). The observation that *CCA1* RNA level can be induced by brief red and far-red illumination shows that the light induction of *CCA1* in etiolated seedlings is mediated by phytochrome, most probably by the very low fluence response of phytochrome A. Interestingly, a recent report (Kolar et al., 1998) has shown that in tobacco the phytochrome-coupled circadian oscillator is regulated by the very low fluence response. Our findings further support the important role of *CCA1* in the phytochrome induction of *Lhcb* gene expression.

CCA1 is also involved in the circadian rhythm of *Lhcb* gene expression. In wild-type plants, the phase of *CCA1* oscillation precedes that of *Lhcb1*1* RNA by about 3 hr. Such a phase relationship is consistent with CCA1 being an activator for *Lhcb* genes and its oscillation driving the oscillation of the *Lhcb* RNAs. This is confirmed by the finding that in the CCA1-ox plants, constitutive expression of CCA1 protein caused constitutive *Lhcb* RNA expression in LL. We have previously shown that CCA1 binds to a 36 bp promoter region of the *Lhcb1*1* gene that is sufficient for circadian rhythm of expression (Carré and Kay, 1995; Wang et al., 1997). Therefore, CCA1 most likely directly regulates *Lhcb* gene expression by binding to the promoter of these genes.

The activity of CCA1 on *Lhcb* gene expression appears to be dependent on light signaling. Whereas the *Lhcb1*1* RNA was maintained at a high level in CCA1-ox plants under LL conditions, it dropped steadily after transferring CCA1-ox plants into constant darkness, even though the *CCA1* RNA was still expressed at a high level. We have also observed that etiolated CCA1-ox plants expressed *Lhcb* RNA at a level similar to that in etiolated wild-type plants (Z.-Y. W. and E. M. T., unpublished data), though they expressed the CCA1 protein at a high level (Figure 2C). These results suggest that the activation of the *Lhcb1*1* gene requires light signal transduction in addition to the presence of CCA1 protein. Light may activate CCA1 either by direct post-translational modification or by regulating activities of other factors that are required for CCA1 function.

Constitutive Expression of CCA1 Affects Multiple Circadian-Regulated Processes

Although CCA1 is a transcription factor for *Lhcb* gene expression, its function is not limited to the regulation of *Lhcb* genes. The fact that its constitutive expression affected flowering time was the first indication that it had a function related to circadian rhythms. Indeed, we have found that the constitutive expression of CCA1 resulted in the alteration of a number of physiological and molecular processes that are regulated by the circadian clock.

It has been known from physiological studies in both plant and animal systems that the circadian clock is involved in photoperiodism (Thomas and Vince-Prue, 1997). Several mutations that affect circadian rhythms have been shown to affect photoperiodic responses also (Hicks et al., 1996; Stirland et al., 1996). *Arabidopsis* is a facultative long-day plant, with flowering promoted by long-day photoperiods. The late flowering phenotype of the CCA1-ox plants is, therefore, most likely a result of disruption of the circadian rhythm that is involved in the normal photoperiodic response.

The longer hypocotyl phenotype of the CCA1-ox plants is also likely to be related to a disruption of a normal circadian clock function. A circadian rhythm in the rate of stem elongation of *Chenopodium rubrum* has been reported (Lecharny and Wagner, 1984). Dowson-Day and Millar have observed that the growth rate of

Arabidopsis hypocotyls also oscillates with a circadian rhythm (M. J. Dowson-Day and A. J. Millar, personal communication), and if this rhythm of growth inhibition were abolished, it might lead to the longer hypocotyl phenotype. The *elf3* mutant has defective circadian clock function and also exhibits a long hypocotyl phenotype (Zagotta et al., 1996). It is, however, yet to be determined whether the circadian rhythm of hypocotyl elongation was disrupted in the CCA1-ox plants.

We have also analyzed expression of genes that are regulated differently from *Lhcb1*1* by the circadian clock. The *CCR2* and *CAT3* RNAs oscillate with a different circadian phase from the *CCA1* and *Lhcb* genes. Furthermore, *CAT3* RNA damps to a high level in DD, whereas the *Lhcb*, *CCA1*, and *CCR2* RNAs damp to low levels. Constitutive expression of *CCA1* affected the rhythmic expression of both *CCR2* and *CAT3* genes as well as of the *Lhcb* gene in both LL and DD conditions. Although the phase relationship is consistent with a possibility that CCA1 may directly suppress the *CCR2* and *CAT3* genes, the fact that the *CCR2* and *CAT3* genes are affected differently from the *Lhcb1*1* in the CCA1-ox plants supports the idea that CCA1 acts less directly on their expression. Compared with the arrhythmic expression of *Lhcb* RNA, the *CCR2* and *CAT3* RNA showed low-amplitude fluctuations in the CCA1-ox plants in LL conditions. The difference in rhythm damping of *CCR2* and *CAT3* also suggests that the clock regulation of these two genes is somewhat different.

Expression of *CCA1* RNA Is Quickly Induced by Phytochrome and Feedback Suppressed by CCA1 Protein

CCA1 RNA can be quickly induced by red and far-red light treatments of etiolated seedlings. Such treatments have been shown to reset the circadian clock through the action of phytochrome (Nagy et al., 1993; Kendrick and Kronenberg, 1994). It is possible that induction of CCA1 mediates the resetting of the circadian clock. Light induction of the *frq* transcript and light-induced TIM protein degradation have been shown to be part of the mechanism by which the circadian clocks are reset by light signals in the corresponding organisms (Crosthwaite et al., 1995; Zeng et al., 1997). Although not yet technically feasible, it will ultimately be of interest to test whether a pulse of CCA1 expression can affect the phase of the circadian rhythms (see Crosthwaite et al., 1995).

The complete suppression of the endogenous *CCA1* gene to the trough level in CCA1-ox plants suggests that the CCA1 feedback repression is responsible for the trough level expression in the normal circadian oscillation of CCA1 in wild-type plants. This feedback suppression is likely mediated by transcriptional regulation. When transgenic plants containing a *CCA1* promoter-*GUS* reporter gene construct were crossed with the CCA1-ox plants, the expression of the *GUS* gene was suppressed (Z.-Y. W. and E. M. T., unpublished data). Although CCA1 binding site sequences are present in the 5′ region of the *CCA1* gene, it is yet to be determined whether CCA1 protein directly suppresses transcription by binding to its own promoter.

Negative feedback regulation is a common feature of the clock genes of *Neurospora* and *Drosophila* and is believed to be responsible for the generation of continued oscillations (Kay and Millar, 1995; Dunlap, 1996). *CCA1* does not share obvious sequence homology with the clock genes in *Neurospora* and *Drosophila*; however, it does negatively regulate its own expression as well as that of *LHY*, and it could potentially serve as part of a feedback regulatory mechanism involved in generating circadian oscillations. We favor the hypothesis that the phytochrome activation and feedback suppression of *CCA1* gene expression are involved in generating its circadian oscillation.

There has been evidence for different oscillators in etiolated seedlings and green plants (see Somers et al., 1998). Millar et al. (1992) detected circadian oscillations of *Lhcb* expression in etiolated tobacco seedlings that had received no light signal, and Kolar et al. (1998) have found evidence for two oscillators regulating *Lhcb* RNA in etiolated tobacco seedlings, only one of which is coupled to phytochrome. It is yet to be determined whether these light-independent circadian rhythms are affected in the CCA1-ox plants and whether CCA1 oscillates in etiolated seedlings.

The accompanying paper describes the characterization of a *CCA1* homolog, the *LHY* gene. The *lhy* mutant, which contains a 35S promoter inserted in the 5′ region of the *LHY* gene and expresses the *LHY* RNA at a higher level than wild type, has similar long-hypocotyl and late-flowering phenotypes as the CCA1-ox plants. The *LHY* gene shares substantial sequence homology with the *CCA1* gene (Schaffer et al., 1998). In addition, both genes are expressed with similar circadian phases and have similar effects on circadian rhythms of gene expression when expressed constitutively. Furthermore, overexpression of *CCA1* can cause similar phenotypes as overexpression of *LHY* while *LHY* expression is suppressed. These results suggest that these two genes have at least some redundant functions. The fact that both *CCA1* and *LHY* genes are suppressed by overexpression of CCA1 also suggests that *CCA1* and *LHY* may be two parallel components in one feedback loop or that the feedback loops of *CCA1* and *LHY* may interact.

The experiments reported here show that *CCA1* has several characteristics expected of a component of the central oscillator or a component that is closely associated with it either on the input or output side. Further experiments are required to understand fully the role it plays in the circadian rhythms of *Arabidopsis*. Studies of the relationship between *CCA1/LHY* and other genes involved in light and circadian clock regulation of plant development will elucidate the biochemical mechanism of circadian oscillation and the regulatory pathways through which plant development is regulated by the natural light conditions.

Experimental Procedures

Expression of CCA1 Protein in Transgenic *Arabidopsis* Plants

The coding sequence of the *CCA1* cDNA (from nucleotide 214 to the 3′ end of clone 25; Wang et al., 1997) was cloned using PCR-aided cloning into the pBI121 vector (Clonetech) at the XbaI/SstI sites downstream of the 35S promoter, yielding the pBCA126 construct. This construct was transformed into *Agrobacterium tumefaciens* strain A2260, and then into *Arabidopsis* plants (Columbia or

WS ecotypes) using the in planta transformation method (Bechtold et al., 1993). Only transgenic lines segregating the kanamycin resistance as a single locus were used in further analysis. Presence of a single-copy T-DNA insert was demonstrated in 11 transgenic lines by genomic Southern blotting following procedures described previously (Wang et al., 1997). The *CCA1* transgene was amplified by PCR from the genomic DNA of one of the transgenic lines and was sequenced and shown to contain wild-type sequence in the coding region.

Plant Growth Conditions

All seeds were imbibed and cold treated at 4°C for 2 days before germination and growth at 22C°–24°C. To grow plants in the dark, the imbibed seeds were exposed to white light for 30 min before growth in a dark room. Plants were grown either on soil Sunshine Mix 3 (Sun Gro Horticulture Inc., Bellevue, WA) or on MS2S medium in petri dishes (Wang et al., 1997). Except when otherwise indicated, light-grown plants were maintained in growth chambers with white light of 150–200 μmol $m^{-2} \cdot s^{-1}$ provided by a combination of fluorescent and incandescent light. Brief red and far-red light treatments of seedlings were as described previously (Wang et al., 1997).

Measurement of Hypocotyl Lengths

Arabidopsis seedlings were grown on MS2S medium plates at 24°C for 5 days. Then, the seedlings were measured using a ruler with resolution of 0.5 mm.

Measurement of the Flowering Times

Arabidopsis plants were grown in 4 × 6 inch pots (about 20/pot) in a growth chamber. The time of bolting was determined as the day when the plant had a bolt of 1 cm. At the same time, the number of rosette leaves was counted.

RNA Gel Blot Analysis

RNA gel blots, synthesis of *UBQ10*, and *CCA1* clone 24 RNA probes were performed as described previously (Wang et al., 1997). To make the RNA probe that detected only the *CCA1* RNA from the endogenous *CCA1* gene but not the RNA from the 35S::CCA1 transgene, a genomic DNA fragment containing nucleotides −68 to +186 relative to transcription start of *CCA1* was amplified by PCR, using primers *CCA132* (5′-GGCAGTTTCTTTTGTAGCCTCGAA) and *T7CCA1* (5′-*TGTAATACGACTCACTATAGAG*AGATGATTCAAT, with T7 promoter sequence in italics). This PCR product was used as a template for synthesis of RNA probe by in vitro transcription.

A similar strategy was used for making the DNA template for synthesis of RNA probes for the *LHY* and *CAT3* genes. The primers for the *LHY* gene were *LHY5* (AACGATGACTTCTCCGGGGAGTT, located at 248 bp upstream of translational start) and *T7LHY* (5′-*TGTAATACGACTCACTATAGGG*TGCAGCTATTCGCTGCTTCA, located 13 bp upstream of translation start, with the T7 promoter sequence in italics), and those for the *CAT3* gene were *CAT3-5* (5′-GGCCATCTCCATATAAGCTCAGTCTATG, located 4 bp downstream of translation stop and primer *T7CAT3* (5′-*TGTAATACGACT CACTATAGGG*TACCACCATAAATCTTAGAA , located 252 bp downstream of translation stop, with the T7 promoter in italics). Hybridization with RNA gel blots was performed as described previously (Wang et al., 1997).

The *CCR2* probe was synthesized from the EcoRI fragment of the *CCR2* cDNA using the random priming method and hybridized with Northern blots as described previously (Carpenter et al., 1994).

Quantitative RT-PCR

To quantitate RNA levels by RT-PCR, cDNA fragments of a control gene (*UBQ10*) and the test genes were synthesized by reverse transcription and amplified by PCR in the same reaction tube. Using the *UBQ10-5′* primer (5′-TAAAAACTTTCTCTCAATTCTCTCT) and the *UBQ10-3′* primer (5′-TTGTCGATGGTGTCGGAGCTT), the PCR product from the *UBQ10* cDNA is 111 bp, and that from the genomic DNA is 415 bp (contains a 304 bp intron). The primers for the *Lhcb1*1* gene were the *cab165-5′* (5′-CTCTCACTCACAAGTTAGTC ATA, *Lhcb1*1* gene specific) and the *cab-3′* (5′-GCAACAGTCTTCCT CATTGTCA-3′) oligonucleotides. The primers for the *CCR2* genes were the *CCR2-5′* (5′-GCTCTTGAGACTGCCTTCGCTC-3′) and *CCR2-3′* (5′-CTCGTTAACAGTGATGCTACGG-3′) oligonucleotides. For detection of the PCR products in polyacrylamide gels, the 5′ primers were labeled with ^{32}P using ^{32}P-γATP and T4 polynucleotide kinase.

Total RNA (5 μg) was treated with 1 μl RQ1 RNase-free DNase (Promega). Then, the enzyme was inactivated by adding EDTA to 3 mM and heating at 100°C for 10 min. The DNase-treated RNA (0.5 μg) was then annealed with 10 nmol each of the *UBQ10-3′* primer and the *cab-3′* or *CCR2-3′* primers. The reverse transcription (RT) was performed by adding dNTP to 1 mM, M-MLV buffer (Promega) to 1× concentration, and M-MLV reverse transcriptase (Promega) to 5U/μl and H_2O to make the final volume 10 μl. The mixtures were incubated at 42°C for 1 hr and then heated at 95°C for 10 min. Then, 15 μl of a PCR mixture (1.7× Tfl PCR buffer [Promega], 2 mM $MgCl_2$, 0.67 μM each of two of the ^{32}P-labeled 5′ primers [*UBQ10-5′* and *cab165-5′*, or *UBQ10-5′* and *CCR2-5′*], and 0.05 U/μl Tfl DNA polymerase [Promega]) was added to each RT reaction. PCR was performed for 16 cycles of 94°C for 45 s, 55°C for 1 min, and 72°C for 1 min. PCR products were separated on a 6% polyacrylamide gel and analyzed using a phosphorimager (Molecular Dynamics, Sunnyvale, CA). The signals of the *Lhcb1*1* or *CCR2* PCR products were normalized by the signals of the *UBQ10* PCR products. In a control experiment where various known amounts of plasmid DNA were used as PCR templates, the ratio between the PCR product of *Lhcb1*1* and *UBQ10* had a linear relationship with the ratio of input amount of *Lhcb1*1* and *UBQ10* DNA (data not shown).

For quantitation of *CCA1* RNA using competitive RT-PCR, total nucleic acid was used, and the PCR product from the cDNA was normalized with that from the genomic DNA, which included a 87 bp intron and therefore was easily distinguished from the product from cDNA. To isolate total nucleic acid, 150 μl of extraction buffer (50 mM Tris [pH 8.3], 150 mM NaCl, 10 mM EDTA, 1% sarcosine) and 300 μl of PCI (phenol/chloroform/isoamyl alcohol, 24:24:1) were added to about 300 mg ground frozen tissue and mixed by vortexing for 30 s. The aqueous phase was reextracted with PCI, and nucleic acid was precipitated by adding 0.1 vol of 3 M sodium acetate (pH 5.2) and an equal volume of isopropanol. *CCA1* cDNA was synthesized by RT (10 μl reaction containing: 1 μg total nucleic acid, 10 nmol *CCA1#2* primer (5′-TTCCYGCAGAGTTTTCTCTT, nucleotides 699–679 of the *CCA1* cDNA), 1 mM dNTP, 1× M-MLV buffer and 50 U M-MLV reverse transcriptase). At the end of RT reaction, 15 μl PCR mixture (see above) containing ^{32}P-labeled *CCA1#1* primer (5′-AAGCGTAAACCAAACAATC) was added, and PCR was performed for 25 cycles of 94°C for 45 s, 55°C for 1 min, and 72°C for 1 min. A standard curve constructed with a constant amount of the genomic *CCA1* clone and increasing amounts of the cDNA clone showed linear correlation between the ratio of input cDNA and genomic DNA and the ratio between their PCR products (data not shown). Similar results were also obtained when samples of a repeat experiment were analyzed by RNA gel blotting.

Western Blotting Analysis of the CCA1 Protein

A polypeptide containing amino acid residues 83–608 of CCA1 was expressed as a glutathione-S-transferase (GST) fusion and purified using a glutathione-agarose affinity column (Wang et al., 1997). This protein was used for immunizing rabbits to obtain polyclonal antisera (carried out by R. B. Sargeant, Ramona, CA). Antibodies were purified from serum using an affinity column made of purified GST-CCA1 fusion protein immobilized on activated Sepharose beads (Sigma), following the protocols of Harlow and Lane (1988). Antibodies were eluted from the affinity column with 100 mM glycine and 100 mM triethylamine.

Arabidopsis protein samples were prepared following Damerval et al. (1986) with some modifications. In brief, tissue ground in liquid nitrogen was precipitated in cold acetone containing 10% trichloroacetic acid (TCA) and 0.07% β-mercaptoethanol (β-ME) (4 ml/1 g powdered tissue) for 1 hr at −20°C, and the insoluble material was collected by centrifugation at 14,000 × g for 15 min. The pellet was washed four times with acetone/0.07% β-ME and then dried under vacuum. Protein was dissolved in 1× Laemmli SDS sample buffer, separated on an 8% SDS-polyacrylamide gel (SDS-PAGE), and then transferred to nitrocellulose membrane. The blots were incubated with the purified anti-CCA1 antibody and peroxidase-conjugated

goat anti-rabbit IgG (Sigma), and developed with the ECL reagent following the instruction of the manufacturer (Amersham). The CCA1 protein was identified as a band that was detected by immune sera from three rabbits but not the preimmune sera of them, increased in most of the CCA1-ox transgenic plants compared to wild-type plants, had similar mobility on SDS-PAGE as the full-length CCA1 protein expressed in *E. coli* or produced by in vitro translation, and was undetectable in a line of plants in which the *CCA1* gene was mutated by a T-DNA insertion (R. Green, Z.-Y. W., and E. M. T., unpublished data). The images of immunoblots were imported into a computer using a digital camera (Kodak DCS 420) and quantitated using the NIH Image 1.60 program.

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