

The protein kinase CK2 is involved in regulation of circadian rhythms in *Arabidopsis*

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A wide range of processes in plants, including expression of certain genes, is regulated by endogenous circadian rhythms. The circadian clock-associated 1 (CCA1) and the late elongated hypocotyl (LHY) proteins have been shown to be closely associated with clock function in *Arabidopsis thaliana*. The protein kinase CK2 can interact with and phosphorylate CCA1, but its role in the regulation of the circadian clock remains unknown. Here we show that plants overexpressing CKB3, a regulatory subunit of CK2, display increased CK2 activity and shorter periods of rhythmic expression of CCA1 and LHY. CK2 is also able to interact with and phosphorylate LHY *in vitro*. Additionally, overexpression of CKB3 shortened the periods of four known circadian clock-controlled genes with different phase angles, demonstrating that many clock outputs are affected. This overexpression also reduced phytochrome induction of an *Lhcb* gene. Finally, we found that the photoperiodic flowering response, which is influenced by circadian rhythms, was diminished in the transgenic lines, and that the plants flowered earlier on both long-day and short-day photoperiods. These data demonstrate that CK2 is involved in regulation of the circadian clock in *Arabidopsis*.

Circadian rhythms are driven by endogenous biological clocks that regulate many biochemical, physiological, and behavioral processes in a wide variety of organisms. According to current models, circadian clocks regulating these rhythms consist of input pathways, a central oscillator, and output pathways (1–3). Oscillators are thought to generate rhythms by a transcription-translation negative feedback loop (4–8). Studies in cyanobacteria, *Neurospora*, *Drosophila*, and mouse have found that both positive and negative elements that activate and inhibit the transcription of clock genes are required to maintain the loop. In addition, posttranscriptional and posttranslational regulation play important roles in circadian clocks in *Drosophila* and *Neurospora* (7–10). The oscillator can be entrained by input pathways from environmental cues such as light and temperature, and, in turn, regulates specific cellular events such as expression of clock-controlled genes (1–3).

Until recently, little was known about the molecular mechanisms of circadian clocks in plants (11–13). In *Arabidopsis thaliana*, the *toc1* mutation affects the period of many circadian rhythms (14, 15). Although the corresponding gene has not yet been cloned, it is thought that *TOC1* encodes a component of the oscillator. The *ELF3* gene is proposed to act in the input pathway (16). Two Myb-related genes, circadian clock-associated 1 (*CCA1*) and late elongated hypocotyl (*LHY*), have also been identified as potential clock genes (17, 18), and *CCA1* was found to act as a transcription factor for *Lhcb* gene expression (19). Expression of *CCA1* and *LHY* oscillates with a circadian rhythm. Constitutive expression of *CCA1* was shown to abolish several distinct circadian rhythms, suppress its own expression as well as that of *LHY*, and delay flowering substantially (17). Lack of *CCA1* in a T-DNA insertion mutant line shortened the periods of *LHY* and other clock-controlled genes (20). Overexpression of *LHY* also caused arrhythmic expression of clock-controlled genes, reduction of its own expression, and photoperiod insensitivity of flowering (18). These data suggest that both *CCA1* and

LHY may encode components of regulatory negative feedback loops closely associated with the central oscillator.

CKB3, a regulatory (β) subunit of the protein kinase CK2, was identified as a protein that can interact with *CCA1* (21). CK2 is a Ser/Thr kinase that is expressed ubiquitously, and the holoenzyme consists of two catalytic α - and two regulatory β -subunits (22–24). CK2 α -subunit itself has some catalytic activity. The β -subunits have been found to stimulate the catalytic activity of the α -subunit, stabilize the α -subunit, and, in some instances, change the substrate specificity of the α -subunit *in vitro*. *Arabidopsis* has at least three genes encoding β -subunits (21). CKB3 and other β -subunits of CK2 interact specifically with *CCA1* both in the yeast two-hybrid system and *in vitro*. Recombinant CK2 can phosphorylate *CCA1 in vitro*. Furthermore, *Arabidopsis* plant extracts contain a CK2-like activity that affects the formation of a DNA-protein complex containing *CCA1*. These results suggest that CK2 can modulate *CCA1* activity, and that CK2 may play a role in the regulation of the circadian clock (21).

We have now tested directly whether CK2 can affect circadian rhythms in *Arabidopsis*. We show that *CKB3*-overexpressing plants display increased CK2 activity and shorter periods of rhythmic expression of *CCA1* and *LHY*. Overexpression of *CKB3* also shortens periods of four other circadian clock-controlled genes and alters timing of flowering. Thus, CK2 is apparently involved in regulation of the circadian clock in *Arabidopsis*.

Materials and Methods

Generation of *CKB3*-Overexpressing *Arabidopsis*. A *Bgl*III-*Hinc*II fragment containing a c-myc encoding sequence in front of *CKB3* was subcloned into the pBI121 vector (CLONTECH). This construct was used to transform *Agrobacterium tumefaciens* strain A2260 and then *Arabidopsis* plants (Columbia ecotype) by using the *in planta* transformation procedure, as described (17). Transgenic lines were selected on MS2S plates (20) containing 50- μ g/ml kanamycin.

Northern Blot and Reverse Transcription-PCR (RT-PCR) Analyses. Total RNA isolation, and RNA blot analyses were performed as described (17). For RT-PCR, 10 μ g of total RNA were treated with RQ1 RNase-free DNase (Promega) and the first-strand cDNA was synthesized as described (17). The product of the first-strand synthesis was then used for PCR to amplify 140-bp *CKB3* cDNA with the primers CKB3F1 (5'-ACAAGGAACG-TAGTGGAGGAGGTG) and CKB3B3 (5'-AACCCTAGAT-GTGGTGGTGGGAAG). As an internal control, primers UBQ10-5' and UBQ10-3' were used to amplify 111-bp *UBQ10* cDNA, as described (17). The resultant PCR fragments were separated on a 2% agarose gel, blotted, and hybridized with 32 P-labeled probes.

Abbreviations: GST, glutathione-S-transferase; L:D, light:dark.

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Western Blot Analyses. Protein extracts were obtained by grinding 10-day-old seedlings in 100 μ l of 3 x SDS-sample buffer [180 mM Tris-HCl (pH 6.8)/6% SDS/30% glycerol/7.5% 2-mercaptoethanol], boiling this mixture for 5 min, and saving the supernatant after centrifugation for 15 min at 14,000 \times *g*. Protein concentration was measured with a protein assay reagent (Bio-Rad). Western blotting and immunostaining were performed by using anti-c-myc monoclonal antibody 9E10 (25), as described (17). Immunoblot analyses of the CCA1 protein were carried out as described (17).

CK2 Activity Assays. Frozen seedlings (100 mg) were ground and extracted with 100 μ l of extraction buffer [50 mM Tris-HCl (pH 7.5)/15 mM MgCl₂/0.1 M KCl/0.25 M sucrose/10% glycerol/1 mM phenylmethylsulfonyl fluoride/protease inhibitor mixture (Boehringer Mannheim), phosphatase inhibitor mixture/14 mM 2-mercaptoethanol]. After centrifugation at 14,000 \times *g* for 15 min, the supernatant was saved, and protein concentration was measured. CK2 assays were carried out at 37°C with 200 μ M CK2 specific peptide substrate RRRDDDSDDD (Boehringer Mannheim) in 25 μ l of CK2 buffer, as described (26).

In Vitro Binding Assays and Kinase Assays. GST-fusion proteins, CKA1, CKB1, CKB3, and ³⁵S-labeled CKB3 were prepared as described (21). ³⁵S-labeled CKB3 was mixed with GST, GST-CCA1, or GST-LHY immobilized on glutathione-agarose beads. Bound proteins were analyzed as described (21). For kinase assays, GST or GST-LHY was incubated with recombinant CK2 subunits or without them in the presence of [γ -³²P]ATP. The phosphorylated samples were analyzed as described (21).

Measurement of the Hypocotyl Lengths. Seedlings were grown on MS2S plates for 5 days. Hypocotyl lengths of seedlings were measured by using a digital camera (Kodak DCS 420) and the National Institutes of Health IMAGE program.

Measurement of Flowering Times. Plants were grown under long-day [light:dark (L:D) 16:8] or short-day (L:D 8:16) conditions, as described (17). The number of total leaves (including cauline leaves on the first inflorescence stem) was counted for 10–20 plants on the day the first flower opened.

Results

Isolation of CKB3-Overexpressing Lines. To examine whether CK2 plays a role in the regulation of the circadian clock, we created transgenic *Arabidopsis* plants overexpressing CKB3. The coding region of the CKB3 cDNA tagged with a c-myc encoding sequence was placed under the control of the strong cauliflower mosaic virus 35S promoter, and the resultant chimeric gene was transformed into *Arabidopsis* plants. Among 16 transgenic lines, each of which had a single site of insertion, two transgenic lines designated ox18 and ox41 were used for further analysis. Amounts of CKB3 transcript in the fourth generation of homozygous CKB3-overexpressing (CKB3-ox) plants were approximately 20 times higher than those in wild-type plants (Fig. 1A). Fig. 1B shows that the transgenic plants contained appreciable amounts of the c-myc-tagged CKB3 protein. Measurement of CK2 activity in whole-cell extracts showed that the transgenic lines exhibited a 1.7-fold increase in CK2 activity (Fig. 1C). This increase is comparable to results in fission yeast in which substantial overexpression of a CK2 β -subunit resulted in 2- to 3-fold increase in kinase activity and caused severe phenotypes (27).

Circadian Expression of CCA1 and LHY Genes Is Altered in CKB3-ox Plants. We next examined whether CKB3 overexpression affected circadian expression of the CCA1 and LHY genes. Oscillations in expression of these genes were robust in wild-type plants when

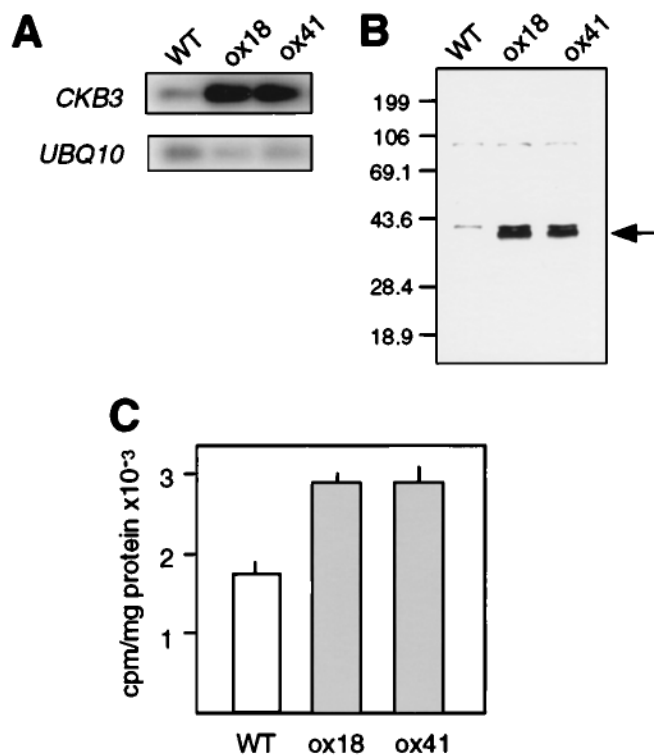


Fig. 1. Overexpression of CKB3 increases CK2 activity. (A) Quantitative reverse transcription-PCR analysis of CKB3 and UBQ10 transcript levels in wild-type (WT) and independent homozygous transgenic lines (ox18 and ox41) after 14 days' growth under L:D 16:8 photoperiods. The UBQ10 transcript levels were used as an internal control. (B) Western blot analysis of myc-CKB3 levels in transgenic lines grown as in A. The c-myc-tagged CKB3 protein (arrow) was detected with anti-c-myc antibody. Other bands are nonspecific. Molecular size markers are given (Left) in kDa. (C) CK2 activity in plant extracts prepared from wild-type and CKB3-ox lines. Plants were grown for 20 days under continuous white light. The data shown are means of two independent experiments for each line, with the range of the measurements indicated.

plants were transferred from growth in L:D 12:12 cycles into continuous light, reaching maximum levels at around subjective dawn and minimum levels at around subjective dusk. Fig. 2A and B show that the periods of CCA1 and LHY RNAs were shortened by about 4 hr in the CKB3-ox plants, and no differences in the amplitude of the rhythms were seen. Fig. 2C shows that the period of the CCA1 protein oscillation was also shortened. Thus, overexpression of CKB3 affected circadian rhythms of these two genes that are closely associated with the circadian clock.

CK2 Can Interact with and Phosphorylate LHY in Vitro. Because LHY is closely related to CCA1 both structurally and functionally (18, 19), we tested the possibility that CK2 can also interact with and phosphorylate LHY. Fig. 3A shows that, like CCA1, LHY could bind to CKB1 and CKB3 efficiently and specifically *in vitro*. Furthermore, Fig. 3B shows that CKA1, the α -subunit of CK2, could phosphorylate LHY, and adding either CKB1 or CKB3 enhanced this phosphorylation. These data are consistent with the idea that CK2 can interact with and phosphorylate both CCA1 and LHY in *Arabidopsis*.

Overexpression of CKB3 Affects Circadian Control of Output Genes. If the function of both CCA1 and LHY is closely associated with a central oscillator and this is altered by increased CK2 activity, the period lengths of the circadian rhythms of output genes should also be changed to reflect those of the CCA1 and LHY RNA rhythms. We therefore tested whether overexpression of

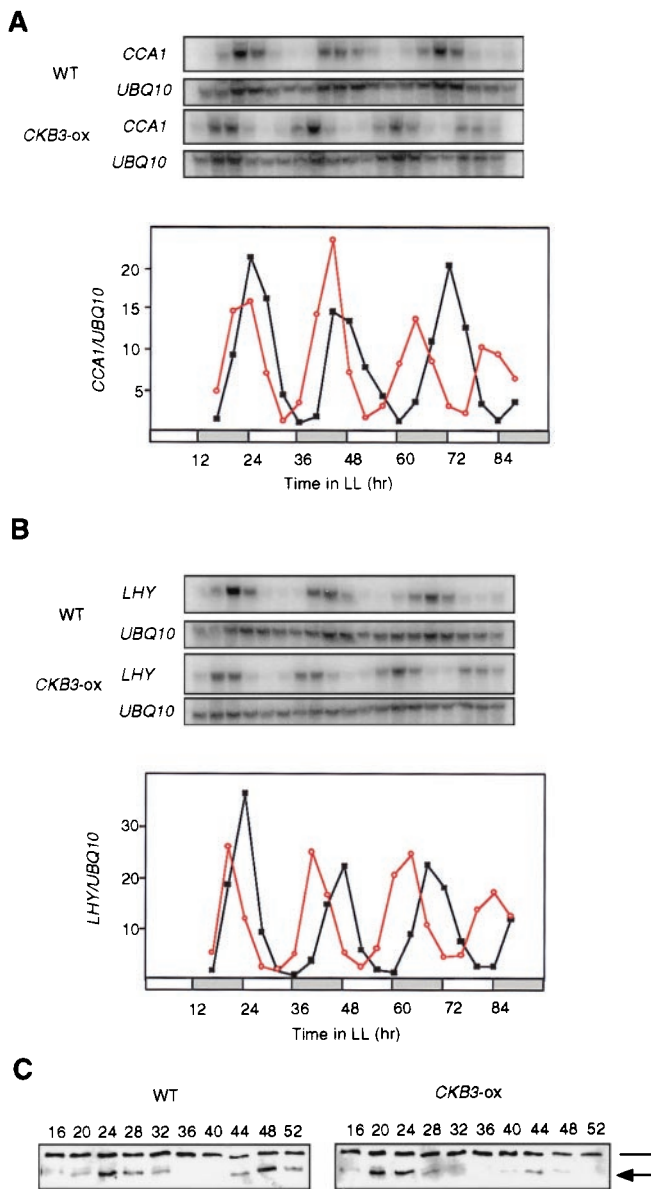


Fig. 2. Overexpression of *CKB3* shortens periods of *CCA1* and *LHY* circadian oscillations. (A) Northern blot analysis of circadian oscillation of *CCA1* transcripts in wild-type and *CKB3-ox* (line ox18) plants. Plants were grown for 12 days in L:D 12:12 photoperiods, then transferred to continuous light after lights-on of day 13. A representative autoradiogram is shown (Upper). (Lower) Quantitation of one experiment. Values were normalized to the lowest value of the wild-type samples. Closed squares, wild type; open circles in red, *CKB3-ox*. The bar represents the subjective light conditions for the wild-type plants. Experiments were performed three times with similar results. The same results were also obtained in another *CKB3-ox* line (ox41). (B) Circadian oscillation of *LHY* RNA in wild-type and *CKB3-ox* plants. *LHY* transcripts in the same RNA samples were analyzed as in A. (C) *CCA1* protein levels in wild-type and *CKB3-ox* (line ox18) plants. Proteins extracted from the same tissue used for RNA preparation were analyzed by Western blotting and detected with anti-*CCA1* antibody. The arrow and line indicate *CCA1* and nonspecific cross-reacting proteins, respectively. Experiments were done twice with similar results.

CKB3 affected genes representing different rhythmic outputs of the circadian clock. In wild-type plants *Lhcb1*1* and *CAT2* RNAs normally peak around subjective dawn and during the subjective day, whereas *CAT3* and *CCR2* RNAs peak around the subjective dusk. Fig. 4 shows that overexpression of *CKB3* had

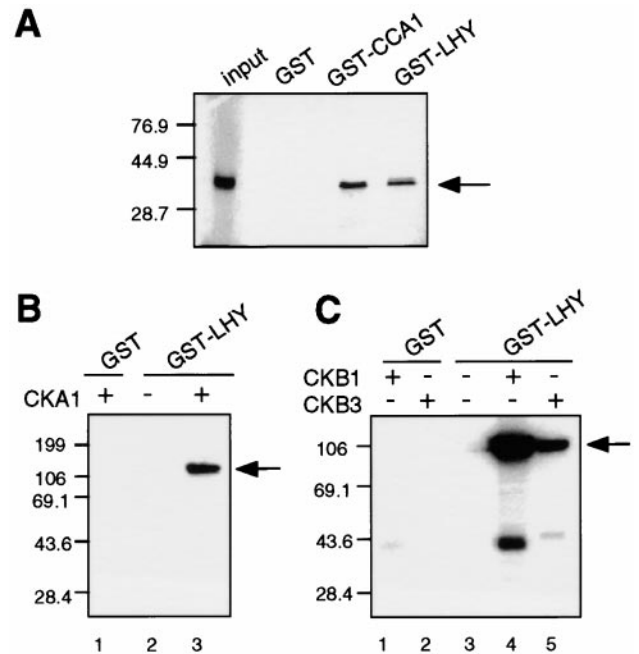


Fig. 3. CK2 can interact with and phosphorylate *LHY* *in vitro*. (A) Interaction of *LHY* with *CKB3* *in vitro*. The input lane represents 10% of the ^{35}S -labeled *CKB3* used, and the other lanes show the amount of bound *CKB3*. The arrow indicates the position of the *CKB3* protein. Molecular size markers are given (Left) in kDa. (B) Phosphorylation of *LHY* by CK2 *in vitro*. GST (lane 1) or GST-*LHY* (lanes 2 and 3) was incubated with 280 ng of CKA1 (lanes 1 and 3) or without CKA1 (lane 2) in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The arrow indicates the position of the GST-*LHY* protein after electrophoresis. (C) CK2 β -subunits enhance phosphorylation of *LHY* by CKA1. GST alone (lanes 1 and 2) or GST-*LHY* (lanes 3–5) was incubated with 14 ng of CKA1 and 35 ng of *CKB1* (lanes 1 and 4) or *CKB3* (lanes 2 and 5) or without β -subunits (lane 3). The arrow indicates the position of the GST-*LHY* protein.

the same effect on the circadian expression of these genes as it did on the *CCA1* and *LHY* RNA rhythms. Although it did not alter the amplitudes, it shortened the periods of the rhythms by about 4 hr. These results suggest that the circadian rhythms of the clock-associated (*CCA1* and *LHY*) and output genes are regulated in a similar way, and that CK2 is involved in this regulation.

Other Effects of *CKB3* Overexpression. We found no apparent effect of *CKB3* overexpression on the overall growth of the plants. Because hypocotyl elongation in *Arabidopsis* seedlings has been shown to occur with a circadian rhythm (28), we examined whether hypocotyl lengths were affected in the transgenic seedlings. We found no differences when seedlings were grown in continuous white light or dark conditions. The average hypocotyl lengths of plants grown in continuous white light were: wild type, 2.19 ± 0.09 mm; *CKB3-ox*, 2.23 ± 0.14 mm (line ox18) and 2.06 ± 0.85 mm (line ox41); in the dark: wild type, 11.6 ± 0.28 ; *CKB3-ox*, 11.3 ± 0.30 mm (line ox18) and 11.6 ± 0.28 mm (line ox41).

Because *CCA1*, a potential substrate of CK2 *in vivo*, is involved in the phytochrome induction of *Lhcb* genes (19), we tested whether this response was altered in the *CKB3-ox* plants. Fig. 5 shows that the phytochrome induction of the *Lhcb1*1* gene was reduced compared with wild-type seedlings. This result is consistent with the observation that antisense expression of a CK2 α -subunit gene increased the phytochrome induction of *Lhcb* RNA under similar conditions (29).

A developmental response in which circadian rhythms are involved is photoperiodic flowering (30). We found that *CKB3* overexpression did affect the photoperiodic induction of flow-

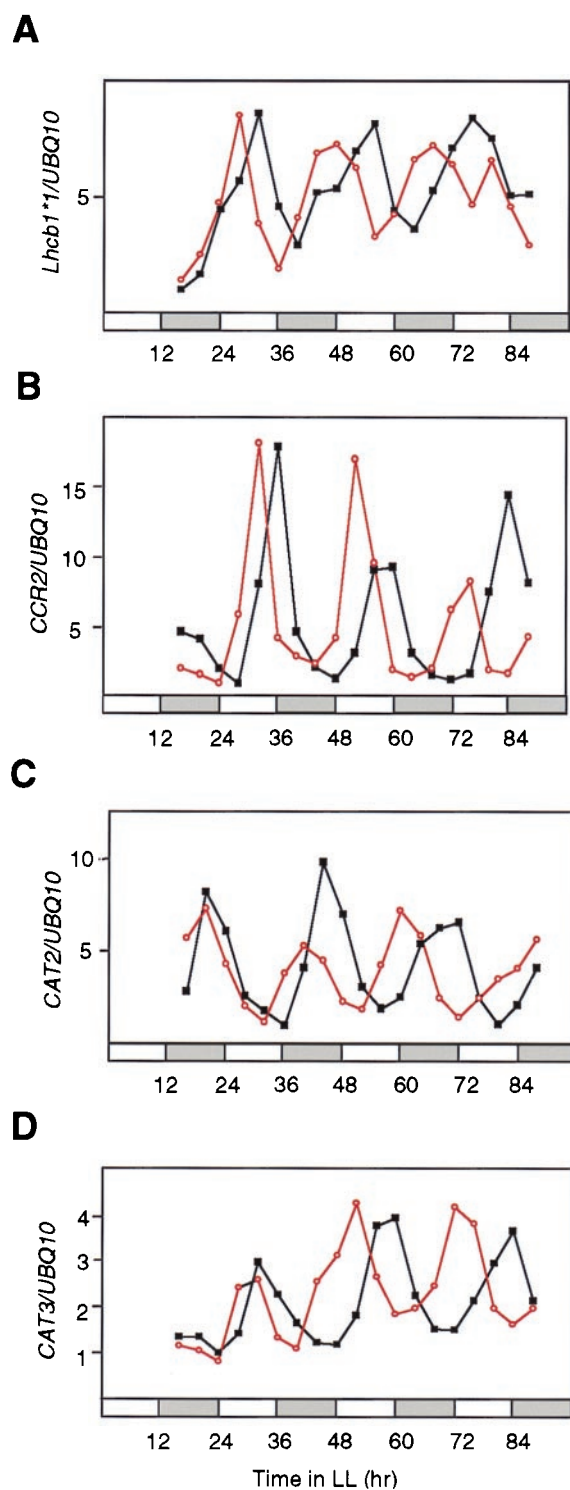


Fig. 4. Periods of output genes are shortened in the *CKB3-ox* transgenic plants. Total RNA was isolated from wild-type and *CKB3-ox* (line ox18) plants grown as in Fig. 2, and expression of *Lhcb1*1* (A), *CCR2* (B), *CAT2* (C), and *CAT3* (D) RNAs was analyzed. Closed squares, wild type; open circles in red, *CKB3-ox*. The bar represents the subjective light conditions for the wild-type plants. Similar results were obtained in three experiments and with another *CKB3-ox* (ox41) line.

ering. Table 1 shows that *CKB3-ox* lines flowered significantly earlier than wild type both in long-day and short-day conditions ($P < 0.001$ for both). Thus, *CKB3* overexpression caused a

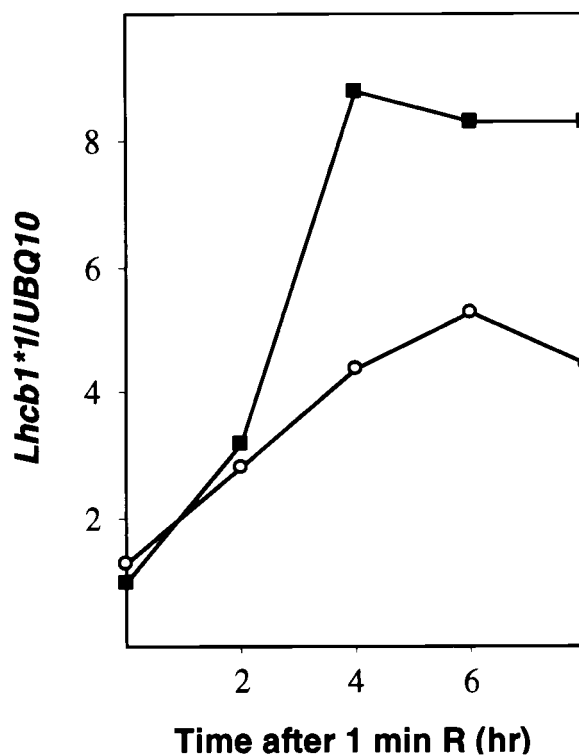


Fig. 5. Overexpression of *CKB3* reduces phytochrome induction of *Lhcb1*1*. Northern analysis of *Lhcb1*1* in 5-day-old etiolated plants after a 1-min exposure to red light. Closed squares, wild type; open circles, *CKB3-ox* (line ox41). Experiments were done twice with similar results.

diminished photoperiodic flowering response, and although we cannot rule out other possibilities, this effect may well be a result of the altered clock function in *CKB3-ox* plants.

Discussion

Overexpression of *CKB3* increased the CK2 activity in plants by about 1.7-fold relative to wild type. This outcome is similar to that seen in other organisms (27, 31). The increase observed for the enzyme activity was much less than the increase in *CKB3* RNA. One possible explanation for this observation is that there may be limited amounts of free α -subunits that can form holoenzyme with the additional *CKB3*.

The *CKB3-ox* lines showed shorter periods of circadian oscillations of *CCA1* and *LHY* RNAs as well as those of several output genes. These findings demonstrate that CK2 is involved in regulating the endogenous circadian clock. Additionally, the timing of flowering was altered. We have also observed that the circadian regulation of leaf movement is affected in the *CKB3-ox* lines (unpublished data). Interestingly, although CK2 can phosphorylate a wide range of substrates, overexpression of *CKB3* did not affect other visible aspects of growth and development of the

Table 1. Timing of flowering in wild-type and *CKB3-ox* plants

	Long days	Short days
Wild type	14.2 \pm 0.2	50.3 \pm 1.6
<i>CKB3-ox18</i>	12.1 \pm 0.2	28.1 \pm 1.0
<i>CKB3-ox41</i>	12.0 \pm 0.2	26.9 \pm 0.9

Flowering time is expressed as total leaf number (rosette leaves plus cauline leaves on the first inflorescence stem) at the time of the opening of flowers. Values are mean \pm SE. Experiments were done three times under long-day and twice under short-day conditions with similar results.

plants. It is possible that components of the circadian clock are more sensitive to increased CK2 phosphorylation than other CK2 substrates, or perhaps CKB3 interacts with clock components in a specific fashion. Further work will be necessary to understand fully the role of CK2 and its subunits in the circadian rhythms of plants.

Some of the characteristics of the *CKB3*-ox lines are similar to those of *toc1* mutant plants, which also exhibit shorter periods of output gene expression and a reduced photoperiodic flowering response but normal hypocotyl elongation (14, 15). Two classes of mutants that affect the input pathway, *det1* and plants overexpressing *phyA* or *phyB*, also have a short-period phenotype (32, 33). However, morphological phenotypes that are associated with these lines are absent in the *CKB3*-ox and *toc1* plants. Furthermore, in contrast to the higher *Lhcb1*1* expression seen in *det1* and the *phyA/B*-overexpressing lines, induction of *Lhcb1*1* in *CKB3*-ox plants by brief red illumination was reduced compared with wild type. We conclude that CK2 affects components that are part of the central oscillator itself or closely associated with it.

Our finding that CK2 can interact with and phosphorylate both CCA1 and LHY *in vitro* is consistent with the idea that CK2 might modulate the circadian clock by direct interaction with and/or phosphorylation of the CCA1 and LHY proteins. Although we have found that *in vitro* the phosphorylation of CCA1 itself did not affect its binding to the *Lhcb1*3* gene (21), we do not know what the consequences of CCA1 and/or LHY phosphorylation might be *in vivo*. In both *Drosophila* and *Neurospora*, phosphorylation of clock components is thought to play a role in their turnover (4–8). It is of interest that a clock gene of

Drosophila, *double-time* (*dbt*), was recently cloned and found to encode a protein closely related to human casein kinase I ϵ . The *dbt* protein can interact with the clock component PER both *in vitro* and in *Drosophila* cells (34). *dbt* mutations alter the periods of behavioral rhythms and molecular oscillations of clock components PER and TIM and also affect PER phosphorylation and stability (35). Although both casein kinase I ϵ and CK2 are Ser/Thr kinases that do not require a second messenger as a cofactor, these kinases are structurally dissimilar, suggesting that these enzymes did not evolve from a common ancestral enzyme.

The evidence presented here leads us to suggest that direct interaction with and/or phosphorylation by CK2 might affect the activity, stability, and/or cellular localization of CCA1 and LHY. The fact that the periods of *LHY* and several output genes were also shortened in a *CCA1*-null line (20) is consistent with this idea. We cannot exclude the possibility that clock components other than or in addition to CCA1 and LHY are affected. It is also possible that overexpression of *CKB3* may sequester a proportion of the endogenous CCA1 and LHY, and that effects caused by *CKB3* overexpression might be independent of other CK2 subunits and of CK2 catalytic activity. However, the results presented here clearly demonstrate that, whatever its interacting partners and/or substrates are *in vivo*, the protein kinase CK2 is involved in the function of the circadian clock in *Arabidopsis*.

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