

CK2 phosphorylation of CCA1 is necessary for its circadian oscillator function in *Arabidopsis*

Xavier Daniel, Shoji Sugano[†], and Elaine M. Tobin[‡]

Department of Molecular, Cell, and Developmental Biology, University of California, Los Angeles, CA 90095-1606

Communicated by Joanne Chory, The Salk Institute for Biological Studies, La Jolla, CA, January 8, 2004 (received for review October 8, 2003)

The circadian clock controls numerous physiological and molecular processes in organisms ranging from fungi to human. In plants, these processes include leaf movement, stomata opening, and expression of a large number of genes. At the core of the circadian clock, the central oscillator consists of a negative autoregulatory feedback loop that is coordinated with the daily environmental changes, and that generates the circadian rhythms of the overt processes. Phosphorylation of some of the central oscillator proteins is necessary for the generation of normal circadian rhythms of *Drosophila*, humans, and *Neurospora*, where CK1 and CK2 are emerging as the main protein kinases involved in the phosphorylation of PER and FRQ. We have previously shown that in *Arabidopsis*, the protein kinase CK2 can phosphorylate the clock-associated protein CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) *in vitro*. The overexpression of one of its regulatory subunits, CKB3, affects the regulation of circadian rhythms. Whether the effects of CK2 on the clock were due to its phosphorylation of a clock component had yet to be proven. By examining the effects of constitutively expressing a mutant form of the *Arabidopsis* clock protein CCA1 that cannot be phosphorylated by CK2, we demonstrate here that CCA1 phosphorylation by CK2 is important for the normal functioning of the central oscillator.

The 24-h periodicity of circadian rhythms enables organisms to coordinate their activities with the external light/dark cycles by anticipating the coming of dawn or dusk. Circadian rhythms in plants include movement of organs, such as leaves and petals, stomata opening, hypocotyl growth, sensitivity of floral induction to seasonal day length changes, and expression of a large number of genes (for review, see ref. 1). Circadian clocks are divided conceptually into three parts: the input signaling pathways, the central oscillator that is itself composed of an autoregulatory negative feedback loop, and the output signaling pathways from the oscillator to various clock-controlled processes. In *Arabidopsis*, two Myb-related transcription factors, CCA1 and its closely related homolog LATE ELONGATED HYPOCOTYL (LHY) are considered to be part of the central oscillator (2–8). The phenotypes of the *timing of cab 1 (toc1)* mutants and *TOC1*-overexpressing plants also indicate a role for the *TOC1* gene product in the central oscillator (5, 9, 10). Taken together, these results suggest the existence of a regulation loop between CCA1/LHY and TOC1 that could account for the central oscillator of the circadian clock in *Arabidopsis*.

We previously identified CKB3 as a CCA1-interacting protein (11). CKB3 is a regulatory (β) subunit of protein kinase CK2 in *Arabidopsis*. CK2 is a Ser/Thr protein kinase that is expressed ubiquitously and is highly conserved. CK2 consists of two catalytic (α) and two regulatory (β) subunits, forming an $\alpha_2\beta_2$ heterotetrameric holoenzyme. CK2 can phosphorylate a wide variety of substrates including transcription factors (12). Phosphorylation of transcription factors has been reported to modulate their DNA-binding activity, cellular localization, stability, and interaction with other proteins (13–15). We previously showed that recombinant CK2 can phosphorylate CCA1 *in vitro* and that plant extracts contain a CK2-like activity that can phosphorylate CCA1 *in vitro* (11). Moreover, the DNA-binding activity of CCA1 from plant extracts requires phosphorylation by

CK2 (11). Plants overexpressing CKB3 (CKB3-ox plants) are affected in the circadian rhythms of gene expression: the period of CCA1 and LHY mRNAs, as well as that of output genes, is shortened from 24 h to 20 h (16). These results indicate that CCA1 phosphorylation by CK2 is important for the normal functioning of the circadian clock in *Arabidopsis*.

Phosphorylation of some of the components of the central oscillator is an important mechanism for regulating the circadian clock in *Neurospora*, *Drosophila*, and humans and affects their subcellular localization and turnover (17–21). CK2 has been shown to be involved in regulating the circadian clock in *Drosophila* (22, 23) and to be an essential component of the *Neurospora* circadian clock (18, 24). Further work on *Neurospora* suggested that FRQ phosphorylation by CK2 was differentially involved in the clock regulation of two different output genes, indicating distinct clock control by the FRQ protein, depending on its CK2 phosphorylation state (25).

Here, by the analysis of transgenic plants overexpressing a novel mutant form of CCA1 that cannot be phosphorylated by CK2, we show that the circadian clock maintains its normal functioning when the overexpressed CCA1 protein does not undergo CK2 phosphorylation. Because we previously showed that overexpression of the wild-type form of CCA1 abolishes circadian rhythms (2), we propose that CCA1 phosphorylation by CK2 is necessary for its function in the central oscillator of *Arabidopsis*.

Materials and Methods

Plasmid Constructs for GST Fusion Proteins. CCA1 deletion mutants were made by digesting pGEX-CCA1 (11) and by religating them with oligonucleotides containing either start or termination codons. To obtain pGEX-mCCA1 (mCCA1 is mutated CCA1) from pGEX-CCA1, amino acid substitution mutants were made by PCR with oligonucleotides containing the substitutions, and mutations were confirmed by DNA sequencing. In detail, the substitutions were T13G and G15T for S5A; T16G and T18C for S6A; T1291G and G1293T for S431A; A1294G, G1295C, and T1296A for S432A; A1297G, G1298C, and T1299C for S433A; and T1450G for S484A.

Recombinant Proteins. GST-CCA1 and GST-mCCA1 fusion proteins were overexpressed in *Escherichia coli* and were purified as described (11).

In Vitro CK2 Phosphorylation Assays. *In vitro* phosphorylation assays were performed as described (11).

Plant Growth Conditions. *Arabidopsis thaliana* plants, ecotype Columbia, were used in these experiments. All seeds were

Abbreviations: DG, 2,3-diphosphoglycerate; LL, continuous light; mCCA1, mutated CCA1.

[†]Present address: National Institute of Agrobiological Sciences, 2-1-2 Kan-nondai, Tsukuba, Ibaraki 305-8602, Japan.

[‡]To whom correspondence should be addressed. E-mail: etobin@ucla.edu.

© 2004 by The National Academy of Sciences of the USA

imbibed and cold-treated at 4°C for 4 days before germination, and grown on soil at 22–24°C.

Production of Plants Overexpressing mCCA1. The entire mCCA1-coding region was cloned into the pBI121 vector (BD Clontech) under the dependence of the CaMV 35S promoter and the NOS terminator. The expression cassette 35S::mCCA1:NOS was subcloned into pHY-BAR, a Basta-resistance binary vector (gift from C. Lin, University of California, Los Angeles). This plasmid was used to transform *Agrobacterium tumefaciens* strain C58 with a heat/cold treatment. *Arabidopsis* plants were transformed according to ref. 26. Transgenic lines were selected by spraying a 1:1,000 dilution of Basta on 2-week-old plants. Homozygous T3 lines containing a single T-DNA insertion were used.

Generation of LHY-Specific Antibodies. LHY-specific polyclonal antisera were raised in rabbits against a GST-LHY protein fusion where the MYB DNA-binding domain of LHY was deleted (IMGEX, San Diego) and used at a dilution of 1:5,000.

Protein Analyses. Plant protein extractions, Western blotting analysis, and protein quantification for CCA1, mCCA1, and LHY were performed as described (2).

Measurement of Flowering Times. Plants were grown on soil in growth chambers under long days (16 h of light/8 h of dark) or short days (8 h of light/16 h of dark) conditions, as described (2). The total number of leaves (including cauline leaves on the first inflorescence stem) was counted for 20 plants on the day the plant had a bolt of 1 cm.

RNA Extractions and RT-PCR. RNA extractions and RT-PCR reactions were performed as described (2). The primers used for the *UBQ10*, *CAB2*, and *CCR2* genes are as described (2). The primers used for the endogenous *CCA1* gene were *CCA1-EF* (5'-GATTTCTCCATTTCCTAGC) and *CCA1-ER* (5'-CTCCAGACGAATTTGTCTCC). PCR products were analyzed as described (2). For accuracy, each reaction was performed three times from two independent experiments.

Yeast Two-Hybrid Protein–Protein Interaction Assays. All assays were performed according to the manufacturer's instructions. The entire coding regions for CCA1, mCCA1, and CKB3, and deletion mutants for CCA1 and mCCA1, were cloned into the plasmids provided in the Matchmaker kit (BD Clontech). Fusion proteins were made between the activation domain of the B42 protein (AD) and CCA1 or mCCA1 full-length or deletion proteins, and between the DNA-binding domain of the LexA protein (LexA) and the full-length CKB3 or CCA1 proteins.

Electrophoretic Mobility-Shift Assays (EMSA). GST-CCA1 and GST-mCCA1 were incubated with 0.1 ng of end-labeled A2 fragment of the *Arabidopsis Lhcb1*3* gene (27) with 0.5 μg of poly(dI-dC) at 25°C for 15 min. Plant extracts were incubated in preincubation buffer (50 mM Tris-HCl, pH 7.5/2 mM MnCl₂/5 mM DTT/0.1 mM EDTA/0.01% Brij 35) at 30°C for 45 min, then incubated with the A2 probe with 1 μg of poly(dI-dC). DG (2,3-diphosphoglycerate, an inhibitor of both CK1 and CK2 protein kinases) and λ phosphatase treatments have been described (11). The EMSA buffer and the electrophoresis conditions have been described (27). DNA–protein complexes were detected by autoradiography.

Results

Identification of CK2 Phosphorylation Sites in CCA1 and Synthesis of mCCA1, Which Cannot be Phosphorylated by CK2. To investigate the importance of CCA1 phosphorylation by CK2 for its function in

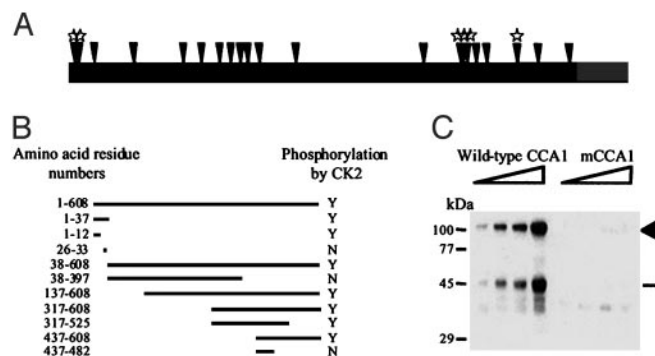


Fig. 1. Identification of CK2 phosphorylation sites in the CCA1 protein and synthesis of mCCA1, which cannot be phosphorylated by CK2. (A) Putative CK2 phosphorylation sites in CCA1, and mutations of serine to alanine in mCCA1. Black arrowheads indicate putative CK2 phosphorylation sites (S/TXXD/E) in CCA1. White stars indicate the six serine residues that were mutated into alanine residues in mCCA1. (B) Schematic representation of the phosphorylation of CCA1 deletion mutants by CK2 *in vitro*. Mutants are designated by their corresponding amino acid residue numbers in full-length CCA1. Y, phosphorylated; N, not phosphorylated. (C) Autoradiograph of SDS/PAGE analysis of GST-CCA1 and GST-mCCA1 after *in vitro* CK2 phosphorylation assays. Increasing amounts of GST-CCA1 or GST-mCCA1 were incubated with recombinant CK2 in the presence of [γ -³²P]ATP. The arrow points to full-length GST-CCA1 or GST-mCCA1. The lower bands indicated by the dash could be degradation products of GST-CCA1.

the circadian clock, we produced a mutant form of the CCA1 protein that cannot be phosphorylated by CK2. We identified the sites in CCA1 that are phosphorylated by CK2 *in vitro*. As diagrammed in Fig. 1A, CCA1 protein contains 21 putative CK2 phosphorylation sites (S/TXXD/E). We made GST fusion proteins harboring N-terminal and/or C-terminal deletion mutants of CCA1 and tested which could be phosphorylated by CK2 *in vitro* (Fig. 1B). Among them, both an N-terminal fragment (amino acids 1–12) and a C-terminal fragment (amino acids 317–525) of CCA1 were found, indicating that these fragments contained residues that were actual targets of CK2 *in vitro* (Fig. 1B). Using mass spectroscopy, we confirmed Ser-5 and -6, as well as one or more of four additional serine residues (Ser-431, -432, -433, and -484) as being phosphorylated by CK2 *in vitro* (data not shown). We substituted nucleotides in CCA1 so that these six serine residues were changed into alanine residues to make a new protein, mCCA1. We performed *in vitro* CK2 phosphorylation assays with increasing amounts of GST-CCA1 or GST-mCCA1 proteins as substrates. The results shown in Fig. 1C demonstrate that these substitutions virtually eliminated mCCA1 phosphorylation by CK2 *in vitro*.

Generation of Plants Overexpressing mCCA1. To compare the consequences of the overexpression of CCA1 and mCCA1 on circadian rhythms, we produced lines of plants overexpressing mCCA1, and identified a line (mCCA1-ox plants) that produces as much mCCA1 as CCA1-ox o34 produces wild-type CCA1 (2). We checked that as in the case of CCA1-ox plants, mCCA1-ox plants show a constant and high level of protein from the construct, as detected by Western blot using anti-CCA1 antibodies, in every condition that plants were grown (data not shown). Fig. 6A, which is published as supporting information on the PNAS web site, compares the levels of mCCA1 protein in four of the mCCA1-ox lines (A–D) to the wild-type, *cca1-1*-null and four CCA1-ox lines. At a time when the CCA1 protein is not detected in the wild-type plants [36 h after transfer of plants to continuous light (LL)], mCCA1-ox lines A–D show large amounts of mCCA1 protein. The mCCA1-ox line D expresses as much mCCA1 as the CCA1-ox o34 line expresses CCA1. We

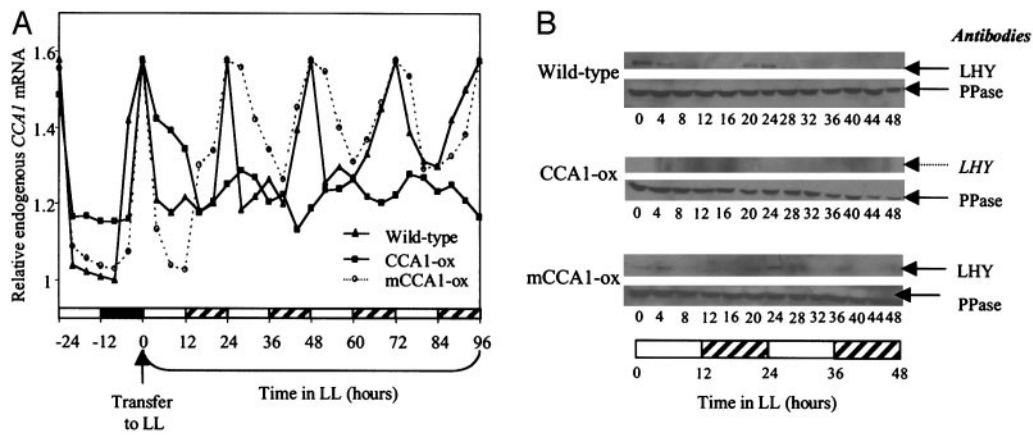


Fig. 2. Unlike overexpression of *CCA1*, overexpression of *mCCA1* does not abolish circadian rhythms in the central oscillator. Wild-type (filled triangles), *CCA1-ox* (filled squares), and *mCCA1-ox* (open circles, dotted line) plants were entrained in light/dark (LD) conditions (12 h of light/12 h of dark) and transferred to constant light (LL). Samples were collected every 4 h and submitted to RNA and protein extractions. (A) Expression of endogenous *CCA1* measured by RT-PCR. The relative levels of endogenous *CCA1* mRNA were normalized to the lowest value of the wild-type samples. Each reaction was performed three times from two independent experiments with similar results. (B) *LHY* protein levels in wild-type, *CCA1-ox*, and *mCCA1-ox* plants measured by Western blot. Pyrophosphatase (PPase) was used as a loading control. Experiments were performed two times with similar results. The solid arrows indicate the location of both *LHY* and PPase proteins. The dotted arrow indicates where *LHY* protein is expected in *CCA1-ox* plants. White and black bars indicate light and dark periods, respectively, in LD. The white and the hatched bars indicate light and subjective dark periods, respectively, in LL.

chose this line for further studies. The expression level of the introduced genes in these two latter lines matches the peak expression level of *CCA1* in the wild-type plants, indicating that in *CCA1-ox* plants, the major effect of overexpression of *CCA1* is more likely to take place at times when *CCA1* is at its trough in wild-type plants.

Unlike Overexpression of *CCA1*, Overexpression of *mCCA1* Does Not Abolish Circadian Rhythms in the Central Oscillator. Plants overexpressing *CCA1* show no circadian rhythms, including arrhythmia in the expression of the endogenous *CCA1* gene, as well as the repression of its transcription to a level matching its trough level in wild-type plants (2). To test whether *CCA1* phosphorylation by CK2 is involved in this negative feedback loop, we monitored the expression of the endogenous *CCA1* gene in wild-type, *CCA1-ox*, and *mCCA1-ox* plants. Fig. 2A shows that under constant light, the period length, phase, and amplitude of endogenous *CCA1* mRNA circadian oscillations in *mCCA1-ox* plants were very similar to those in wild-type plants, whereas constitutive expression of *CCA1* suppressed the rhythmic expression of endogenous *CCA1*. Additionally, we note that the decay in the endogenous *CCA1* gene mRNA is slowed in *mCCA1-ox* plants. *CCA1-ox* plants also display abolished cir-

cadian rhythms of the closely *CCA1*-related gene *LHY*, and the *LHY* mRNA and protein levels match the trough levels in the wild-type plants (2). Fig. 2B shows that under LL, protein levels of *LHY* exhibited the same circadian oscillations in *mCCA1-ox* plants as in wild-type plants, whereas its production was severely reduced in *CCA1-ox* plants. These results demonstrate that *CCA1* phosphorylation by CK2 is necessary for *CCA1* overexpression to repress the functioning of the central oscillator.

Unlike Overexpression of *CCA1*, Overexpression of *mCCA1* Does Not Abolish Circadian Rhythms of Output Genes. If *CCA1* is a component of a negative feedback loop that is an integral part of the circadian clock, the circadian rhythms of output genes should reflect those of *CCA1*. To test the existence of a functioning circadian clock in *mCCA1-ox* plants, we monitored the circadian expression of two output genes, *CAB2* and *CCR2*, which differ in their phase of expression, indicating that they are regulated at distinct times by the circadian clock. Overexpression of the wild-type form of *CCA1* abolishes the circadian oscillations of *CAB2* and *CCR2* mRNAs (2). In contrast, Fig. 3 shows that under constant light conditions, both *CAB2* and *CCR2* transcripts continue to oscillate in *mCCA1-ox* plants with the same phase and period as in the wild-type plants, although the

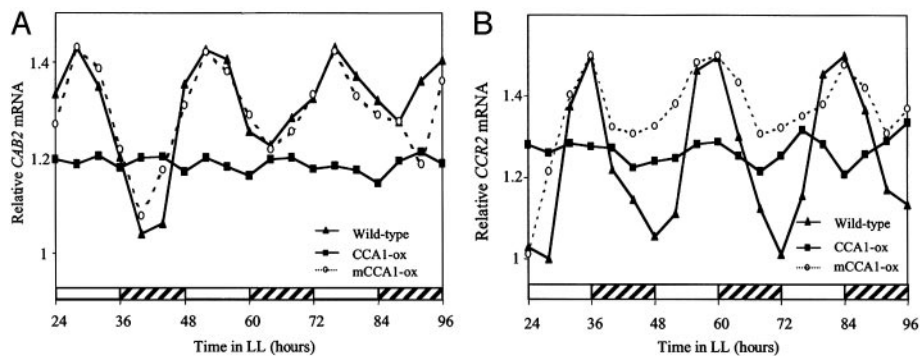


Fig. 3. Unlike overexpression of *CCA1*, overexpression of *mCCA1* does not abolish circadian rhythms of output genes. Plants were treated as in Fig. 2. The relative levels of *CAB2* (A) and *CCR2* (B) mRNA were normalized to the lowest value of the wild-type samples. For both panels, the white and the hatched bars indicate light and subjective dark periods in LL, respectively. Each reaction was performed three times from two independent experiments with similar results.

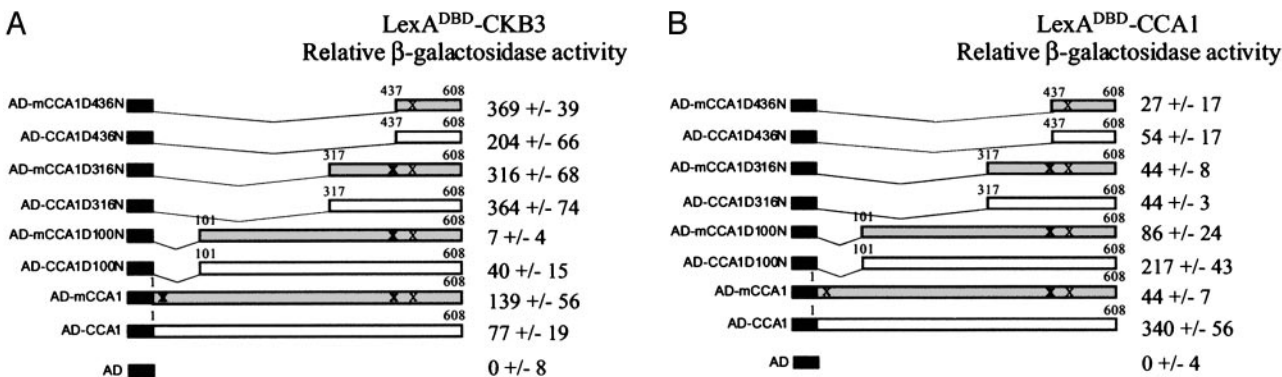


Fig. 4. Mutations in mCCA1 do not alter interaction with CKB3 but do alter interactions with CCA1. All experiments were done according to the manufacturer's instructions for the Matchmaker kit (BD Clontech). For both A and B, the left side illustrates CCA1 (open bars) or mCCA1 (gray bars) fusions to the B42 transcription activation domain (black boxes) that were constructed in pB42AD and introduced into yeast strain EGY48 with a plasmid expressing full-length CKB3 (A) or full-length CCA1 (B) fused to the LexA DNA-binding domain. CCA1 and mCCA1 N-terminal deletions fused to the B42 transcription activation domain are indicated by numbers. Black crosses indicate mutations in mCCA1. The right side shows the corresponding β -galactosidase activities of CPRG (chlorophenol red- β -D-galactopyranoside) assays, relative to the negative control arbitrarily set to zero. The values are the average of six individual colonies from a representative experiment \pm SE.

amplitude in *CCR2* mRNA oscillations were somewhat reduced. Thus, CCA1 phosphorylation by CK2 is essential for the constitutively produced protein to abolish the circadian rhythms of the output genes.

Phenotypes That Have Multiple Determinants Are Not as Profoundly Affected as the Rhythms Themselves. Overexpression of mCCA1 does not delay flowering as much as does overexpression of CCA1. Photoperiodic flowering is influenced by the circadian clock in *Arabidopsis* (2, 3, 28–30). Overexpression of CCA1 substantially delays flowering under short days and long days (2). As shown in Fig. 7, which is published as supporting information on the PNAS web site, mCCA1-ox plants also show a delayed flowering phenotype under short days and long days, although the delay is less pronounced than in CCA1-ox plants. Fig. 6B shows that the extent of the delay in flowering of mCCA1-ox plants is proportional to the amount of mCCA1 protein produced, as previously described for CCA1-ox plants (2), demonstrating that both proteins are functional proteins.

Overexpression of mCCA1 does not reduce viability as much as does overexpression of CCA1. Circadian rhythms provide organisms a way to anticipate daily and seasonal environmental changes (31). The adaptive advantage of circadian rhythms has been demonstrated in cyanobacteria, antelope ground squirrel (*Ammospermophilus leucurus*), and chipmunks (*Tamias striatus*) (32–34), and was recently demonstrated in *Arabidopsis* (35, 36). Plants that have lost the anticipation of dawn because of abolished circadian rhythms, such as CCA1-ox plants or plants overexpressing LHY (LHY-ox), show a greatly reduced viability under very short days (4 h of light/20 h of dark) (35). Fig. 8A, which is published as supporting information on the PNAS web site, shows that under long days, mCCA1-ox plants display the same survival rate as wild-type plants and CCA1-ox plants. Although the flowering time of mCCA1-ox plants and CCA1-ox plants was significantly delayed compared to wild-type plants, overall growth and development were similar in these conditions. Fig. 8B shows that mCCA1-ox plants did not show the same impairment of survival as CCA1-ox plants did under very short days. Whereas CCA1-ox plants survive very poorly under these conditions (<5% of plants surviving 4 weeks after sowing), mCCA1-ox plants display a much higher survival rate (55% of plants surviving 4 weeks after sowing). In addition, mCCA1-ox plants look as healthy as wild-type plants in very short days, and eventually flowered, although much later than wild-type plants, whereas CCA1-ox plants grew very poorly and quickly started

dying. This finding is consistent with previous observations showing that despite the loss of circadian oscillations for the endogenous *CCA1* gene, CCA1-ox plants remain responsive to light under light/dark photoperiods but are not able to anticipate dawn (35). Fig. 2A shows that under light/dark conditions and unlike CCA1-ox plants, mCCA1-ox plants have only partially lost this anticipation: the rise of the endogenous *CCA1* gene mRNA before lights are turned on is reduced but not abolished. These results show that this overall adaptive advantage of a functioning circadian clock also depends on CCA1 phosphorylation by CK2.

Overexpression of mCCA1 leads to a long hypocotyl/long petiole phenotype, as does overexpression of CCA1. Inhibition of hypocotyl growth occurs with a circadian rhythm (37). As a result, the disruption of circadian rhythms can lead to a long hypocotyl phenotype, as seen in the *elf3* mutant and CCA1- and LHY-ox plants (2, 3, 37). There are as yet no such data for petiole growth, although both CCA1- and LHY-ox plants show elongated petioles (data not shown). As for the flowering phenotype described earlier, the increase in length seen in the long petiole/long hypocotyl phenotypes of CCA1-ox plants is proportional to the amount of CCA1 protein in the different lines (data not shown and ref. 2). We found the same correlation concerning the length of petioles and hypocotyls in mCCA1-ox plants (data not shown). These results suggest that CK2 phosphorylation is dispensable for the CCA1 function in the regulation of petiole and hypocotyl elongation.

Mutations in mCCA1 Do Not Alter Interaction with CKB3 but Do Alter Interactions with CCA1. Phosphorylation of transcription factors by CK2 has been shown to modulate their ability to interact with proteins (14). To further explore the differences in phenotypes obtained in mCCA1-ox plants compared to CCA1-ox plants, we addressed the ability of mCCA1 to interact with CKB3 and the wild-type form of CCA1. We constructed N-terminal deletions of CCA1 and mCCA1 to identify the domains of CCA1 and mCCA1 that are responsible for the interactions. Fig. 4A shows that mCCA1 can interact physically with CKB3; the domain responsible for the interaction with CKB3 is in both CCA1 and mCCA1 comprised between amino acids 317 and 608. Fig. 4A also shows that the six point mutations in mCCA1 do not prevent the interaction with CKB3. Hence, a weaker or absent interaction between mCCA1 and CKB3 cannot account for the mCCA1-ox phenotype.

The formation of dimers of clock components is known to be a key point in the function of the central oscillator in *Neurospora*

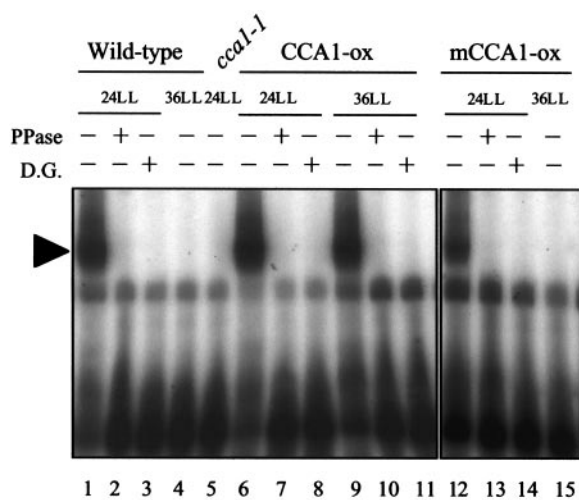


Fig. 5. Phosphorylation by CK2 is required for the formation of the DNA-protein complex containing CCA1 in plant extracts. Wild-type, CCA1-ox, and mCCA1-ox plants were treated as in Fig. 2. Plant extracts were incubated in the absence (lanes 1, 4, 5, 6, 9, 12, and 15) or presence of λ -phosphatase (PPase) (lanes 2, 7, 10, and 13) or DG (lanes 3, 8, 11 and 14). The arrowhead indicates the DNA-protein complex containing CCA1.

and mammals (38–41). To address the importance of a CCA1/CCA1 homodimer in the regulation of the circadian clock, we performed yeast two-hybrid assays between the full-length CCA1 protein, and full-length or deletions of CCA1 and mCCA1 proteins. Fig. 4B shows that CCA1 can interact physically with itself. A very strong β -galactosidase activity was monitored with two copies of the full-length CCA1 protein. The domain responsible for this interaction is between amino acids 100 and 317. When these experiments were performed with mCCA1, the interaction strength was reduced dramatically; it was 7.7-fold weaker with full-length mCCA1 than with full-length CCA1.

Phosphorylation by CK2 Is Required for the Formation of the DNA-Protein Complex Containing CCA1 in Plant Extracts. Fig. 5 shows that 24 h after transfer to LL (24LL), wild-type plants contain an activity that binds to the A2 fragment but is absent from plant extracts from the *cca1-1*-null line, indicating the presence of CCA1 in this complex. When the plant extracts were treated with λ -phosphatase or with DG, formation of the complex was inhibited. This complex is present in plant extracts from CCA1-ox plants at 24LL, and at a time when it is not detected in the wild-type plants (36LL), showing that the CCA1 DNA-binding activity is constant in CCA1-ox plants. Both λ -phosphatase and DG treatments of CCA1-ox plant extracts abolish the formation of the complex at time points 24LL and 36LL, indicating the dependence on CK2 phosphorylation for the formation of the complex in CCA1-ox plants. The complex is present at 24LL in mCCA1-ox plants. It depends on CK2 phosphorylation because λ -phosphatase and DG treatments abolish it, but it is not present at 36LL. mCCA1 protein is present at high levels at all times (data not shown). Therefore, the complex at 24LL must only contain the wild-type form of CCA1, and not mCCA1. In addition, the absence of the complex at 36LL indicates that mCCA1 cannot be part of the complex. These results show that phosphorylation by CK2 is required for the formation of the DNA-protein complex containing CCA1 in plant extracts.

Discussion

Although there are several examples of phosphorylation by CK2 of clock components in animals and *Neurospora* (22–25), little is

known about this topic in plants. We have shown that CK2 can phosphorylate two components of the central oscillator in *Arabidopsis*, CCA1 and LHY (11, 16). We also showed that the CCA1-DNA-binding activity extracted from plants required phosphorylation by CK2 (11). Moreover, we artificially increased CK2 kinase activity in CKB3-ox plants and linked this overexpression to altered circadian rhythms, where components of the central oscillator (CCA1 and LHY), as well as four output genes (CAB2, CCR2, CAT2, and CAT3), showed shorter mRNA circadian oscillations than in the wild-type plants (16).

Our results here that compare CCA1-ox plants with mCCA1-ox plants show that CCA1 phosphorylation by CK2 is necessary to abolish circadian rhythms in CCA1-ox plants, indicating its importance in CCA1 function in the regulation of the circadian clock in *Arabidopsis*. The endogenous CCA1 gene expression is not altered at the mRNA level by the overexpression of mCCA1 (Fig. 2A); hence, the wild-type form of the CCA1 protein is most probably also oscillating and being phosphorylated by CK2 as in the wild-type line, and therefore normal circadian rhythms are observed (Fig. 3). Meanwhile, the mCCA1 protein is produced at a constant high level, indicating that CCA1 phosphorylation by CK2 is required for its regulation of circadian rhythms. The preliminary data in Fig. 9 and *Supporting Text*, which are published as supporting information on the PNAS web site, show that overexpression of mCCA1 in the *cca1-1* null background leads to the same phenotypes as in the wild-type background. In *Neurospora*, a mutant form of the FRQ protein that affects its phosphorylation by CK2 differentially influences its circadian regulation of two output genes, one being arrhythmic and the other behaving as in the wild-type strain (18). However, the CK2 phosphorylation of this mutant FRQ was not abolished (25), and the differences in regulation could be due to this partial loss of CK2 phosphorylation. Overexpressing the wild-type form of CCA1 or mCCA1 leads to different phenotypes. These disparities are due to the differences in the phosphorylation state of the CCA1 protein. Increased amounts of the phosphorylated form of CCA1 are therefore responsible for abolishing circadian rhythms in CCA1-ox plants, whereas high levels of mCCA1 do not abolish them in mCCA1-ox plants, confirming the importance of *in vivo* CCA1 phosphorylation by CK2 for its circadian oscillator function.

Our results further suggest that CCA1 phosphorylation by CK2 in CCA1-ox plants is not involved in the elongated hypocotyl and petiole phenotype and that it is not necessary for, but enhances the delay in flowering time. Changes in flowering time, as well as hypocotyl growth rate and eventually height, can sometimes be attributed to a disturbed circadian clock (2, 3, 37), but this correlation does not always hold, as previously shown in the case of the *toc1-1* mutant (42, 43). Although we do not know of any evidence other than that presented here concerning the petiole phenotype, it seems logical that as for flowering time and hypocotyl growth, petiole growth is likely to involve other pathways in addition to ones regulated by the circadian clock. Because delayed flowering, long hypocotyl, and long petiole phenotypes are observed both in CCA1- and mCCA1-ox plants, we propose that the phosphorylation of CCA1 by CK2 is not necessary for these phenotypes.

We have considered the possible roles of CCA1 phosphorylation by CK2 for its function in the circadian oscillator. Phosphorylation of transcription factors by CK2 has been shown to influence their interaction with other proteins, DNA-binding activity, turnover rate, and nuclear localization (13–15, 24).

CCA1 interacts with a variety of proteins, including catalytic and regulatory subunits of CK2, LHY, and CCA1 itself (ref. 11, this study, and data not shown). Fig. 4 shows that although the interaction of CCA1 with CKB3 is not influenced by the mutations in mCCA1, they dramatically reduced the strength of the CCA1/CCA1 interaction. In mCCA1-ox plants, the inter-

action between the endogenous CCA1 protein and the mCCA1 protein may not be strong enough to hinder the proper function of CCA1 and to disrupt circadian rhythms. Although we do not yet have direct evidence that CCA1 phosphorylation is taking place or is hindered for mCCA1 in yeast, we had shown that CKB3 from *Arabidopsis* is functional in yeast and can restore CK2 activity from a *ck2* mutant yeast strain (11). The data presented here may indicate a need for CCA1 to be phosphorylated by CK2 to form a homodimer and could partly account for the mCCA1-ox phenotype.

Phosphorylation of transcription factors by CK2 was shown to modulate their DNA-binding activity (13). The DNA-binding activity of CCA1 may be impaired by the mutations in mCCA1, as expected from results in ref. 11 that showed the dependence on CK2 phosphorylation for CCA1 to bind DNA. If this is true, mCCA1 is expected not to bind promoters that are normally targeted by CCA1 after its phosphorylation by CK2. Fig. 5 demonstrates that this is indeed the case for the *Lhcb1*3* promoter and suggests a similar scenario concerning other CCA1 target promoters. These data are consistent with previously published observations. Phosphorylation of recombinant CCA1 was reported not to enhance the CCA1 DNA-binding activity (11). In addition, the DNA-protein complex from plant extracts containing CCA1 migrates slower than the complex using only *E. coli*-produced CCA1, showing that plant extracts contain other factor(s) that are needed for the formation of this complex *in planta*. Hence, comparing recombinant CCA1 and mCCA1 abilities to bind the A2 fragment was not the best route, and we chose to use plant extracts to perform EMSAs.

Phosphorylation of transcription factors can target them for degradation in *Arabidopsis* (44). Degradation of *Drosophila* and

Neurospora PER clock proteins depends on their phosphorylation (18, 21, 45). If CCA1 degradation depends on its phosphorylation by CK2, the degradation rates of the two forms of CCA1 may be different, as phosphorylation by CK2 could for example target CCA1 for degradation.

In mammals and *Drosophila*, phosphorylation of the clock protein PER was shown to be important for its nuclear localization (19, 46). CCA1 accessibility to its target promoters obviously depends on its nuclear localization and could rely on its phosphorylation by CK2. Therefore, only the wild-type form of CCA1 would be able to regulate the transcription of the target genes when translocated to the nucleus, whereas the mCCA1 protein might be sequestered in the cytoplasm. Then, the mCCA1-ox plants would be expected to show differences in transcription of clock-controlled genes compared to the CCA1-ox plants.

One or more of the above possibilities could account for the differences observed between CCA1- and mCCA1-ox plants, and further experiments will be needed to decipher the molecular mechanisms involved. Nonetheless, the dependence on CCA1 phosphorylation by CK2 for its ability to form a homodimer and to bind one of its target promoters have been shown here. Our results demonstrate clearly that CCA1 phosphorylation by CK2 is necessary for the normal functioning of the circadian clock in *Arabidopsis*.

We thank J. Lee, W. Hwong, and L. Chung for excellent technical assistance, and Dr. C. Lin for the gift of the pHY-BAR binary vector. This work was supported by grants to E.M.T. from the U.S. Department of Agriculture (99-35304-8156) and the National Institutes of Health (GM23167).

- Barak, S., Tobin, E. M., Andronis, C., Sugano, S. & Green, R. M. (2000) *Trends Plant Sci.* **5**, 517–522.
- Wang, Z. Y. & Tobin, E. M. (1998) *Cell* **93**, 1207–1217.
- Schaffer, R., Ramsay, N., Samach, A., Corden, S., Putterill, J., Carré, I. A. & Coupland, G. (1998) *Nat.* **393**, 1219–1229.
- Green, R. M. & Tobin, E. M. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 4176–4179.
- Alabadi, D., Oyama, T., Yanovsky, M. J., Harmon, F. G., Más, P. & Kay, S. A. (2001) *Science* **293**, 880–883.
- Alabadi, D., Yanovsky, M. J., Más, P., Harmer, S. L. & Kay, S. A. (2002) *Curr. Biol.* **12**, 757–761.
- Green, R. M. & Tobin, E. M. (2002) *Dev. Cell* **2**, 516–518.
- Mizoguchi, T., Wheatley, K., Hanzawa, Y., Wright, L., Mizoguchi, M., Song, H. R., Carré, I. A. & Coupland, G. (2002) *Dev. Cell* **2**, 629–641.
- Millar, A. J., Carré, I. A., Strayer, C. A., Chua, N. H. & Kay, S. A. (1995) *Science* **267**, 1161–1163.
- Makino, S., Matsushika, A., Kojima, M., Yamashino, T. & Mizuno, T. (2002) *Plant Cell Physiol.* **43**, 58–69.
- Sugano, S., Andronis, C., Green, R. M., Wang, Z. Y. & Tobin, E. M. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 11020–11025.
- Meggio, F. & Pinna, L. A. (2003) *FASEB J.* **17**, 349–368.
- Klimczak, L. J., Schindler, U. & Cashmore, A. R. (1992) *Plant Cell* **4**, 87–98.
- Bek, S. & Kemler, R. (2002) *J. Cell Sci.* **115**, 4743–4753.
- Bontems, S., Di Valentin, E., Baudoux, L., Rentier, B., Sadzot-Delvaux, C. & Piette, J. (2002) *J. Biol. Chem.* **277**, 21050–21060.
- Sugano, S., Andronis, C., Ong, M. S., Green, R. M. & Tobin, E. M. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 12362–12366.
- Ederly, I., Zwiebel, L. J., Dembinska, M. E. & Rosbash, M. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 2260–2264.
- Liu, Y., Loros, J. & Dunlap, J. C. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 234–239.
- Bao, S., Rihel, J., Bjes, E., Fan, J. Y. & Price, J. L. (2001) *J. Neurosci.* **21**, 7117–7126.
- Toh, K. L., Jones, C. R., He, Y., Eide, E. J., Hinz, W. A., Virshup, D. M., Ptacek, L. J. & Fu, Y. H. (2001) *Science* **291**, 1040–1043.
- Ko, H. W., Jiang, J. & Ederly, I. (2002) *Nature* **420**, 673–678.
- Lin, J. M., Kilman, V. L., Keegan, K., Paddock, B., Emery-Le, M., Rosbash, M. & Allada, R. (2002) *Nature* **420**, 816–820.
- Akten, B., Jauch, E., Genova, G. K., Kim, E. Y., Ederly, I., Raabe, T. & Jackson, F. R. (2003) *Nat. Neurosci.* **6**, 251–257.
- Yang, Y., Cheng, P. & Liu, Y. (2002) *Genes Dev.* **16**, 994–1006.
- Yang, Y., Cheng, P., He, Q., Wang, L. & Liu, Y. (2003) *Mol. Cell. Biol.* **23**, 6221–6228.
- Bechtold, N., Ellis, J. & Pelletier, G. (1993) *C. R. Acad. Sci.* **316**, 1194–1199.
- Sun, L., Doxsee, R. A., Harel, E. & Tobin, E. M. (1993) *Plant Cell* **5**, 109–121.
- Nelson, D. C., Lasswell, J., Rogg, L. E., Cohen, M. A. & Bartel, B. (2000) *Cell* **101**, 331–340.
- Roden, L. C., Song, H. R., Jackson, S., Morris, K. & Carré, I. A. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 13313–13318.
- Simpson, G. G. & Dean, C. (2002) *Science* **296**, 285–289.
- Thomas, B. (1998) in *Biological Rhythms and Photoperiodism in Plants*, eds. Lumsden, P. J. & Millar, A. J. (Bios Scientific, Oxford), pp. 151–165.
- Ouyang, Y., Andersson, C. R., Kondo, T., Golden, S. S. & Johnson, C. H. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 8660–8664.
- DeCoursey, P. J., Krulas, J. R., Mele, G. & Holley, D. C. (1997) *Physiol. Behav.* **62**, 1099–1108.
- DeCoursey, P. J., Walker, J. K. & Smith, S. A. (2000) *J. Comp. Physiol.* **186**, 169–180.
- Green, R. M., Tingay, S., Wang, Z. Y. & Tobin, E. M. (2002) *Plant Physiol.* **129**, 576–584.
- Michael, T. P., Salomé, P. A., Yu, H. J., Spencer, T. R., Sharp, E. L., McPeck, M. A., Alonso, J. M., Ecker, J. R. & McClung, C. R. (2003) *Science* **302**, 1049–1053.
- Dowson-Day, M. J. & Millar, A. J. (1999) *Plant J.* **17**, 63–71.
- Crosthwaite, S. K., Dunlap, J. C. & Loros, J. J. (1997) *Science* **276**, 763–769.
- Cheng, P., Yang, Y., Gardner, K. H. & Liu, Y. (2002) *Mol. Cell. Biol.* **22**, 517–524.
- Shearman, L. P., Sriram, S., Weaver, D. R., Maywood, E. S., Chaves, I., Zheng, B., Kume, K., Lee, C. C., van der Horst, G. T., Hastings, M. H. & Reppert, S. M. (2000) *Science* **288**, 1013–1019.
- Kondratov, R. V., Chernov, M. V., Kondratova, A. A., Gorbacheva, V. Y., Gudkov, A. V. & Antoch, M. P. (2003) *Genes Dev.* **17**, 1921–1932.
- Somers, D., Webb, A., Pearson, M. & Kay, S. (1998) *Development (Cambridge, U.K.)* **125**, 485–494.
- Más, P., Alabadi, D., Yanovsky, M. J., Oyama, T. & Kay, S. A. (2003) *Plant Cell* **15**, 223–236.
- Hardtke, C. S., Gohda, K., Osterlund, M. T., Oyama, T., Okada, K. & Deng, X. W. (2000) *EMBO J.* **19**, 4997–5006.
- He, Q., Cheng, P., Yang, Y., He, Q., Yu, H. & Liu, Y. (2003) *EMBO J.* **22**, 4421–4430.
- Vielhaber, E. L., Duricka, D., Ullman, K. S. & Virshup, D. M. (2001) *J. Biol. Chem.* **276**, 45921–45927.