A Role for Protein Kinase Casein Kinase2 α-Subunits in the Arabidopsis Circadian Clock

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Circadian rhythms are autoregulatory, endogenous rhythms with a period of approximately 24 h. A wide variety of physiological and molecular processes are regulated by the circadian clock in organisms ranging from bacteria to humans. Posttranslational modification of clock proteins plays a critical role in generating proper circadian rhythms. Casein Kinase2 (CK2) is an evolutionarily conserved serine/threonine protein kinase composed of two catalytic α-subunits and two regulatory β-subunits. Although most of the molecular components responsible for circadian function are not conserved between kingdoms, CK2 is a well-conserved clock component modulating the stability and subcellular localization of essential clock proteins. Here, we examined the effects of a cka1a2a3 triple mutant on the Arabidopsis (Arabidopsis thaliana) circadian clock. Loss-of-function mutations in three nuclear-localized CK2α subunits result in period lengthening of various circadian output rhythms and central clock gene expression, demonstrating that the cka1a2a3 triple mutant affects the pace of the circadian clock. Additionally, the cka1a2a3 triple mutant has reduced levels of CK2 kinase activity and CIRCADIAN CLOCK ASSOCIATED1 phosphorylation in vitro. Finally, we found that the photoperiodic flowering response, which is regulated by circadian rhythms, was reduced in the cka1a2a3 triple mutant and that the plants flowered later under long-day conditions. These data demonstrate that CK2α subunits are important components of the Arabidopsis circadian system and their effects on rhythms are in part due to their phosphorylation of CIRCADIAN CLOCK ASSOCIATED1.

Biological rhythms with a period close to 24 h are called circadian rhythms and are found in a diverse array of organisms. Circadian systems are complex signaling networks that allow organisms to anticipate and prepare for regular environmental changes, thus providing them with an adaptive advantage (Ouyang et al., 1998; Green et al., 2002; Dodd et al., 2005). The circadian system can be divided conceptually into three parts: inputs that receive environmental cues to entrain the oscillator; a central oscillator that generates self-sustained rhythmicity; and outputs that consist of various rhythmic processes. The core of a circadian system, the central oscillator, generally shares a conceptually conserved mechanism in eukaryotes, consisting of a transcription-translation feedback loop (Dunlap, 1999). Circadian changes in protein subcellular localization, stability, and phosphorylation also contribute to the generation and maintenance of rhythms (Young and Kay, 2001; Mehra et al., 2009).

In the primary autoregulatory feedback loop in Arabidopsis (Arabidopsis thaliana), transcription factors CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) repress the transcription of TIMING OF CAB EXPRESSION1 (TOCI). In turn, TOC1 (also known as PSEUODORESPONDER REGULATOR1 [PRR1]) activates transcription of CCA1 and LHY through CCA1 HIKING EXPEDITION and other unknown mechanisms (Pruneda-Paz et al., 2009). CCA1 and LHY also repress the transcription of genes encoding EARLY FLOWERING3 (ELF3) and ELF4, LUX ARRHYTHMO (LUX; also known as PHYTOCLOCK1), and GIGANTEA (GI), all of which contribute to the positive regulation of CCA1 and LHY expression, constituting the secondary feedback loop (Covington et al., 2001; Liu et al., 2001; Doyle et al., 2002; Hazen et al., 2005; Kikis et al., 2005; Locke et al., 2005; Onai and Ishiura, 2005). In addition, PRR7 and PRR9, two TOC1 homologs, have been suggested to form an additional feedback loop with CCA1 and LHY (Farré et al., 2005; Nakamichi et al., 2010).

Posttranslational modification of clock proteins is essential for generating proper circadian rhythms (Young and Kay, 2001; Mehra et al., 2009). Phosphorylation of oscillator components appears to play a critical role in regulating their function (Liu et al., 2000; Lin et al., 2002; Akten et al., 2003; Daniel et al., 2004; Tamaru et al., 2009; Tsuchiya et al., 2009). Despite the conceptual similarity in clock mechanisms, there is little sequence conservation between clock components of plants, fungi, insects, and animals. One remarkable
exception is the protein kinase CK2 (formerly Casein Kinase2). In *Drosophila*, CK2 directly phosphorylates the core clock component *PERIOD* (*PER*), thereby regulating its nuclear localization and stability (Lin et al., 2002; Akten et al., 2003). CK2 also plays an essential role in the mammalian clock by regulating the nuclear entry of the clock component *BMAL1* (Tamaru et al., 2009) and the protein stability of *PER2* (Tsuchiya et al., 2009). In addition, CK2 phosphorylation of the *Neurospora* central clock component *FREQUENCY* (*FRQ*) regulates period length by determining its protein stability (Liu et al., 2000; Yang et al., 2002, 2003).

CK2 is a Ser/Thr protein kinase that is evolutionarily conserved and ubiquitously expressed in all eukaryotic cells. The CK2 holoenzyme consists of two catalytic α-subunits and two regulatory β-subunits in a tetrameric (α2β2) complex (Litchfield, 2003) that has more than 300 substrates involved in a wide variety of cellular processes (Meggio and Pinna, 2003). In Arabidopsis, there are four α-subunits (A1–A4) and four β-subunits (B1–B4), which show relatively high sequence similarity within the subunits (Salinas et al., 2006). Knockdown expression of the CK2β subunits (CKBs) lengthens period in Arabidopsis protoplasts (Kim and Somers, 2010), and overexpression of CKB3 or CKB4 leads to period shortening in transgenic Arabidopsis (Sugano et al., 1999; Perales et al., 2006). Both CKB3 and CKB4 interact with the central clock component *CCA1*, and phosphorylation of *CCA1* by CK2 is important for its clock function (Sugano et al., 1998; Daniel et al., 2004; Portolés and Más, 2010).

It has been reported that the CK2 α- and β-subunits can function independently of CK2 tetramers (Bibby and Litchfield, 2005). Little is known regarding the role of CK2α subunits in the circadian clock. To examine their function in the clock, we isolated loss-of-function mutants for three nuclear-localized CK2α subunits and generated a *cka1a2a3* triple mutant. The *cka1a2a3* mutations affect various flowering pathways, the pace of the circadian clock, and *CCA1* phosphorylation, suggesting that CK2α subunits are essential clock components that are critical for maintaining the correct period length through their effect on *CCA1* phosphorylation.

**RESULTS**

**Generation of the Arabidopsis cka1a2a3 Triple Mutant**

To determine the biological roles of CK2α subunits in Arabidopsis, we obtained the T-DNA insertion mutants of individual α-subunits from the Arabidopsis Biological Resource Center. In the *cka1* mutant (SALK_073328) and *cka2* mutant (SALK_129331), the T-DNAs are inserted in the eighth and second intron of the corresponding genes, and in the *cka3* mutant (SALK_022432), the T-DNA is inserted in the 5’-untranslated region of the gene (Fig. 1A). Full-length transcripts were not detected in any of the mutants by reverse transcription (RT)-PCR (Fig. 1B), indicating that they are loss-of-function mutants for the three respective α-subunits. We obtained a *cka2a3* double mutant by crossing *cka2* and *cka3*. *cka1* was then crossed with the *cka2a3* double mutant to generate the *cka1a2a3* triple mutant.

**The cka1a2a3 Mutations Affect Flowering Time**

To elucidate the molecular function of CK2α subunits in Arabidopsis, we examined flowering time in *cka* single, double, and triple mutants. Under long-day (LD) conditions, the three *cka* single mutants had a
similar flowering time as wild-type plants and the cka2a3 double mutant displayed a subtle phenotype, flowering slightly later than the wild type (Fig. 2, A and B). The cka1a2a3 triple mutations substantially delayed flowering time, as measured by days to flowering or number of leaves at flowering (Fig. 2, A and B). The cka1a2a3 triple mutant showed the most profound phenotype and was chosen for further characterization. To determine whether the cka1a2a3 mutations affect flowering time through the photoperiodic response, we examined the flowering phenotype under short-day (SD) conditions. Our results revealed that the cka1a2a3 triple mutant displayed a subtle phenotype, flowering slightly later than wild-type plants under SD conditions (Fig. 2C), suggesting that the cka1a2a3 triple mutant has decreased sensitivity to day-length changes.

Flowering time is controlled by four different pathways, including the photoperiodic, autonomous, and vernalization- and gibberellic acid-dependent pathways (Mouradov et al., 2002). To determine the molecular mechanisms of the delayed flowering phenotype of the cka1a2a3 triple mutant, we examined the expression of FLOWERING LOCUS T (FT) and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1), which encode floral integrators (Parcy, 2005). Compared to the wild type, the rhythmic expression of FT and SOC1 were substantially reduced in cka1a2a3 triple mutant plants (Fig. 3, A and B). FLOWERING LOCUS C (FLC), a convergence point of the autonomous and the vernalization pathways, represses flowering through direct binding to FT and SOC1 chromatin to repress their expression (Helliwell et al., 2006). The transcript level of FLC was strongly increased in the cka1a2a3 triple mutant relative to the wild type (Fig. 3C), suggesting that either the autonomous or vernalization pathway could be affected by the cka1a2a3 triple mutations. The cka1a2a3 triple mutant exhibits a day-length-dependent flowering phenotype. To determine whether the cka1a2a3 triple mutations affect flowering through the photoperiodic pathway, we examined the expression of CONSTANS (CO). CO is a key gene in the photoperiodic flowering pathway.

Figure 2. The cka1a2a3 triple mutations affect flowering time. A, Photographs of plants of various genotypes grown for 23 d under LD (16L/8D) conditions. B, Flowering time of seedlings of various genotypes under LD (16L/8D) conditions. C, Flowering time of the wild type and cka1a2a3 triple mutant under SD (8L/16D) conditions. Flowering time is expressed as either days to bolting or rosette leaf number. Data are means ± sd (n = 18–25). Asterisks indicate a significant difference by Student’s two-tail t test (P < 0.05). WT, Wild type.
pathway that accelerates flowering by light-dependent activation of its immediate target gene, FT (Suárez-López et al., 2001; Yanovsky and Kay, 2002; Valverde et al., 2004). We found that the rhythmic expression of CO was significantly reduced in cka1a2a3 plants (Fig. 3D), indicating that the photoperiodic flowering pathway is affected.

The cka1a2a3 Mutations Affect the Period Lengths of Various Circadian Outputs

The photoperiodic flowering pathway is known to be regulated by the circadian clock and many Arabidopsis mutants with aberrant clock function exhibit early- or late-flowering phenotypes. To determine whether the cka1a2a3 triple mutant has a defect in circadian clock function, we examined leaf movement rhythms, a well-established circadian response in Arabidopsis (Hicks et al., 1996). Seedlings were entrained for 10 d with 12 h light/12 h dark (12L/12D) and subsequently transferred to constant light (LL). Wild-type plants exhibited a robust rhythmic movement of primary leaves with a free-running period length of 24.3 ± 0.4 h (Fig. 4, A and B). In cka1a2a3 triple mutant plants, a robust circadian rhythm of leaf movement was observed, but with a free-running period length of 25.9 ± 1.3 h (Fig. 4, A and B), which is approximately 1.5 h longer than that in the wild type. To assess the robustness of the circadian rhythms in individual seedlings, relative amplitude error (RAE) was measured using fast Fourier transform nonlinear least square analysis. RAE values range between 0 and 1, and a smaller RAE indicates a more robust rhythm. cka1a2a3 seedlings had RAE values of approximately 0.2, similar to those of the wild type (Fig. 4B), suggesting that the cka1a2a3 triple mutations cause period lengthening but do not affect the amplitude and robustness of leaf movement rhythms.

To determine the pervasiveness of CK2α subunits function in the circadian clock, the circadian reporter CHLOROPHYLL A/B BINDING PROTEIN2::LUC (CAB2::LUC; Millar et al., 1995; Knowles et al., 2008) was transformed into cka1a2a3 triple mutant plants. Luminescence was examined in wild-type and cka1a2a3 triple mutant plants entrained for 7 d with 12L/12D and then transferred to LL. CAB2::LUC expression oscillated with a period length of 25.0 ± 0.4 h and 26.2 ± 0.2 h in the wild type and cka1a2a3 triple

Figure 3. Transcript abundance of flowering time genes in the wild type and cka1a2a3 triple mutant. Shown are expression levels of FT (A), SOC1 (B), FLC (C), and CO (D) transcripts in wild-type and cka1a2a3 mutant plants grown under LD (16L/8D) conditions. Seven-day-old seedlings sampled at 4-h intervals were analyzed by qRT-PCR. Black circles, wild type; white squares, cka1a2a3. Day and night are denoted by white and black bars, respectively. The data are presented as the mean of two biological replicates ± so. All experiments were done at least twice with similar results.

Figure 4. The cka1a2a3 triple mutations lengthen the free-running period of circadian rhythms under continuous light (LL) conditions. A and B, Assay of circadian leaf movement under LL conditions. A, Normalized positions of primary leaves for the wild type (n = 17) and cka1a2a3 (n = 15) are shown. B, Period length and RAE estimates of the leaf movement rhythms shown in A. C and D, Assay of CAB2::LUC activity under LL conditions. C, Mean bioluminescence traces of groups of approximately 20 seedlings are shown. D, Period length and RAE estimates of the CAB2::LUC bioluminescence rhythms shown in C. Black circles, wild type; white squares, cka1a2a3. Day and subjective night are denoted by white and hatched bars, respectively. All experiments were done at least twice with similar results.
mutant, respectively (Fig. 4, C and D). Consistent with the period lengthening observed in leaf movement rhythms, CAB2::LUC oscillations in the cka1a2a3 triple mutant were observed to be 1 to 1.5 h longer than in the wild type. Robustness similar to leaf movement rhythms was observed in CAB2::LUC rhythms (Fig. 4D). Together, these results show that the cka1a2a3 mutations affect the period lengths of circadian output rhythms (leaf movement and CAB2::LUC activity), indicating that CK2α subunits are involved in regulating period length, rather than amplitude and robustness in the circadian clock.

The cka1a2a3 Mutations Affect Period Lengths of Circadian Expression of Central Oscillator Genes

To determine whether the cka1a2a3 mutations affect the pace of the central oscillator or only a subset of outputs, we examined the circadian expression of the central oscillator genes CCA1, LHY, TOC1, and LUX in the cka1a2a3 triple mutant. The oscillations of expression of all four genes were robust and displayed a longer period length in the cka1a2a3 triple mutant than in wild-type plants (Fig. 5). We also checked the expression of other clock genes, such as PRR7, PRR9, ELF3, and GI, that are proposed to function in the interlocked feedback loops within the central oscillator (McClung, 2006). The cka1a2a3 mutations lengthened the expression period length of all genes examined without affecting the amplitude of their expression (Supplemental Fig. S1), which is consistent with the findings that the cka1a2a3 mutations affect period length but do not alter the amplitude and robustness of the circadian output rhythms such as leaf movement rhythms (Fig. 4). Therefore, CK2α subunits are important in controlling the pace of the Arabidopsis circadian clock.

The cka1a2a3 Triple Mutant Has Reduced CK2 Kinase Activity

To investigate whether aberrant clock function in the cka1a2a3 triple mutant is due to a defect in CK2 kinase activity, we performed a CK2 kinase assay using a CK2-specific peptide substrate and radiolabeled ATP. The cka1a2a3 triple mutant showed an approximately 70% reduction in kinase activity when compared with the wild type (Fig. 1C). Heparin, a specific inhibitor of CK2 (Park et al., 2008), was used in control reactions to demonstrate that the effect was only due to CK2 activity and not other kinases (Fig. 1C). These data suggest that the overall activity of the CK2 holoenzyme is affected by the cka1a2a3 triple mutations.

Phosphorylation of CCA1 Is Reduced in the cka1a2a3 Triple Mutant

In Arabidopsis, CCA1 is a central oscillator component (Wang and Tobin, 1998; Green and Tobin, 1999; Knowles et al., 2008). CCA1 protein phosphorylation by CK2 has been shown to be essential for its proper function in the circadian clock (Daniel et al., 2004). To determine the specific effects of CK2 activity on the phosphorylation of CCA1, we expressed CCA1 as a GST fusion protein in Escherichia coli and performed an in vitro kinase assay using radiolabeled GTP and different amounts of whole-cell plant extracts from light-grown seedlings. We used GTP in this assay (rather than ATP) to limit the activity of non-CK2 kinases in the reaction. Unlike many kinases, CK2 can utilize GTP as a phosphoryl donor nearly as efficiently as it can utilize ATP (Sugano et al., 1998). Plant extracts from wild-type seedlings phosphorylated GST-CCA1 more effectively than extracts from the cka1a2a3 triple mutant (Fig. 6A). We observed a 30% average reduction in the amount of phosphorylated GST-CCA1 in the triple mutant relative to the wild type (Fig. 6B). These data suggest that the cka1a2a3 mutations affect CCA1 phosphorylation in vitro. To examine whether CCA1 phosphorylation in planta is also impaired in the cka1a2a3 triple mutant, immunoblotting was performed using anti-CCA1 antibodies with total extracts from 2-week-old seedlings grown in 12L/12D and harvested at different times. CCA1 protein peaks at 1 h after dawn and decays rapidly within 6 h in wild-type plants (Fig. 7). In the cka1a2a3 triple mutant, a broader peak of CCA1 protein has been detected, which is

Figure 5. The cka1a2a3 triple mutations lengthen the free-running period of central oscillator gene expression. Shown is qRT-PCR analysis of CCA1 (A), LHY (B), TOC1 (C), and LUX (D) expression in wild-type and cka1a2a3 triple mutant plants under LL conditions. Ten-day-old seedlings were entrained in a 12L/12D cycle, transferred to LL, and harvested for 3 d at 4-h intervals. The mean of two biological replicates ± sn is shown. Black circles, wild type; white squares, cka1a2a3. Day and subjective night are denoted by white and hatched bars, respectively. All experiments were done at least twice with similar results.
consistent with the period-lengthening phenotype observed in the cka1a2a3 triple mutant (Fig. 7). We were unable to differentiate the phosphorylated and unphosphorylated form of CCA1 protein. Together, these results suggest that CK2α subunits affect the pace of the circadian clock through their regulation of CCA1 phosphorylation and the timing of CCA1 protein abundance.

DISCUSSION

CK2 is a tetrameric protein kinase formed by two catalytic α-subunits and two regulatory β-subunits. Increasing evidence indicates that localization and interaction of CK2 subunits with other proteins is a dynamic process (Litchfield, 2003; Olsten et al., 2005). In fact, the CK2α monomer exists as an active form independent of β-subunits, and the regulatory β-subunit can modulate substrate specificity and catalytic activity (Sarno et al., 2002; Tamaru et al., 2009). It has also been reported that the regulatory β-subunit can interact with other protein kinases and perform functions independently of CK2 tetramers (Bibby and Litchfield, 2005). Arabidopsis has four catalytic α-subunits and four regulatory β-subunits (Salinas et al., 2006). Studies on the CKBs demonstrate that CKB3 and CKB4 interact with central clock component CCA1 and overexpression of CKB3 or CKB4 causes period shortening in Arabidopsis (Sugano et al., 1999; Perales et al., 2006). Nothing is known regarding the function of the CK2α subunits in the circadian clock and the clock phenotype of loss-of-function mutations in either α- or β-subunits has not been reported in Arabidopsis plants. We isolated T-DNA mutant lines for three CK2α subunits (CKA1, CKA2, and CKA3) that have been shown to be localized in the nucleus (Salinas et al., 2006). We expected that no obvious phenotype would be observed in any single mutant plants (Fig. 2; data not shown) due to the high sequence similarity among them (Salinas et al., 2006). We therefore generated cka2a3 double and cka1a2a3 triple mutants by genetic crosses. The cka2a3 double mutant has a subtle flowering-time phenotype and the cka1a2a3 triple mutant has a profound phenotype, flowering much later than the wild type under LD conditions (Fig. 2, A and B), supporting the idea that functional redundancy exists within this group of subunits. Interestingly, although CK2 participates in a wide variety of cellular processes, the cka1a2a3 triple mutation has no discernable effect on plant growth and development (Fig. 2; data not shown). Measurements of CK2 activity in whole-cell extracts revealed a significant decrease of CK2 activity in the cka1a2a3 triple mutant (Fig. 1C), which amounted to 30% of that of the wild-type plants. Our results are consistent with previous studies with ck2α antisense plants that showed a more than 60% inhibition of kinase activity compared with the wild type and had a low impact on plant growth and development (Lee et al., 1999).

Figure 6. Phosphorylation of GST-CCA1 protein in vitro using whole-cell extracts prepared from wild-type (WT) and cka1a2a3 seedlings. A, Recombinant GST-CCA1 protein was mixed with radiolabeled GTP and varying amounts of whole-cell extracts. Reactions were incubated at 30°C for 10 min. After washing, the reactions were subjected to SDS-PAGE. The gel was Coomassie stained (bottom image) and exposed to a phosphor screen (top image). The numbers above the lanes indicate the amounts of total protein from the whole-cell extracts that were added to the reaction. White arrowhead, Full-length GST-CCA1; asterisk, GST-CCA1 degradation product. The experiment was performed three times with similar results. B, Relative intensities of the full-length GST-CCA1 band (cka1a2a3/wild type) from the phosphor screen image shown in A. Error bars denote the SEM from three independent experiments.

Figure 7. CCA1 protein abundance in wild-type and cka1a2a3 triple mutant plant extracts. Shown are western-blot analysis of total plant extracts and detection with antibody to CCA1. Actin was used as a loading control. Two-week-old seedlings grown in 12L/12D were harvested at different times as indicated. The experiment was performed at least twice with similar results.
Recent studies showed that overexpression of the *ck2α* kinase-inactive mutant resulted in severe growth and developmental defects and eventually lethality (Moreno-Romero et al., 2008). The strong phenotype is probably due to the *ck2α* kinase-inactive mutant interacting and sequestering the endogenous CKBs (Moreno-Romero et al., 2008). Our *cka1a2a3* triple mutant allows us to examine the role of CK2α in the circadian system without affecting the endogenous CKBs and to separate clock defects from growth and developmental defects.

We found that *cka1a2a3* triple mutant plants are impaired in their ability to sense day length, flowering later than wild-type plants when grown under LD conditions. The *cka1a2a3* triple mutant exhibited reduced CO and FT expression, suggesting that CK2α subunits are involved in the photoperiodic flowering pathway for the regulation of floral induction. These results, combined with the finding that a CK2α subunit mediates the photoperiodic flowering response of rice (*Oryza sativa*; Takahashi et al., 2001), demonstrate that CK2α subunits play an important role in the regulation of flowering in both LD and SD plants. The circadian clock interacts with the photoperiodic pathway to regulate seasonal flowering; therefore, mutations that disrupt clock function often affect photoperiodic flowering (Yanovsky and Kay, 2003; Searle and Coupland, 2004). Our results revealed that the *cka1a2a3* triple mutations caused lengthening of the free-running period of various circadian output rhythms (leaf movement and *CAB2::LUC* activity), suggesting that CK2α subunits are important in regulating period length. Moreover, the *cka1a2a3* triple mutations caused period lengthening in the expression of all genes examined, including central clock genes (CCA1, LHY, TOC1, and LUX; Fig. 5) and genes involved in other interlocked feedback loops (PRR7, PRR9, GI, and ELF3; Supplemental Fig. S1). Taken together, these results suggest that CK2α subunits function close to the central oscillator in controlling the pace of the circadian clock.

In *Neurospora*, disruption of CK2α abolishes circadian rhythmicity and results in FRQ hypophosphorylation and elevated FRQ levels (Yang et al., 2002). In *Drosophila*, ck2α homozygote mutants do not live to adulthood and heterozygotes show a lengthened period of behavioral rhythms by 3 h, exceeding that of nearly all heterozygous circadian mutants in *Drosophila* (Lin et al., 2002). Considering the evolutionarily conserved function for CK2 in circadian clocks, the comparably weak phenotype observed in the *cka1a2a3* triple mutant (about 1.5 h longer period) suggests that either the fourth CK2α subunit (CKA4), which has been shown to be localized in the chloroplast (Salinas et al., 2006), could be exported out of the chloroplast to partially complement the loss of three nuclear-localized CKAs or the regulatory CKBs could perform functions independent of CK2 tetramers. It has been shown that CK2 can phosphorylate CCA1 and affect its function in the clock (Daniel et al., 2004). A recent study also showed that CK2 activity interferes with CCA1 DNA binding (Portolés and Más, 2010). Our observation that reduced CCA1 phosphorylation in vitro in the *cka1a2a3* triple mutant (Fig. 6) is consistent with the notion that lower CK2 activity leads to reduced CCA1 phosphorylation, which increases the residence time of CCA1 at the promoters and results in period lengthening. It is known that CK2 phosphorylation of the central clock component FRQ affects its protein stability in *Neurospora* (Liu et al., 2000). It is possible that phosphorylation by CK2 could target CCA1 protein for degradation. A broader peak of CCA1 protein in *cka1a2a3* triple mutant plants (Fig. 7) supports the idea that reduced CCA1 phosphorylation leads to more stable CCA1 protein, which results in period lengthening.

Our findings demonstrate that CK2α subunits play essential roles in the Arabidopsis clock by controlling the pace of the clock and that this control could be mediated by CCA1 phosphorylation. Circadian phenotypic differences between *Drosophila* CK2α and CK2β mutants suggest that catalytic and regulatory CK2 subunits may have distinct physiological roles in clock function. Further studies will be needed to elucidate the detailed mechanisms that regulate CK2 activity in vivo and the relative roles of α- and β-subunits in the Arabidopsis circadian system.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

Arabidopsis (*Arabidopsis thaliana*; Columbia ecotype) was used for all experiments described unless stated otherwise. *cka1* (SALK_073328), *cka2* (SALK_129501), and *cka3* (SALK_022432) were obtained from the Arabidopsis Biological Resource Center. *cka2* and *cka3* were crossed to obtain the *cka2a3* mutant that was then crossed with *ckat* to obtain the *cka1a2a3* triple mutant. Seedlings were grown under a 12 h fluorescent light (100 μmol m⁻² s⁻¹)/12 h dark (12L/12D) photoperiod at a constant temperature of 22°C, unless otherwise stated. All primers used in genotyping can be found in Supplemental Table S1.

**Analysis of Circadian Rhythms**

For the luciferase experiments, Arabidopsis plants homozygous for *cka1a2a3* and the wild type (Columbia) were transformed with the *CAB2::LUC* reporter (Knowles et al., 2008). T2 seedlings from three independent transformed lines were entrained for 6 d under 12L/12D conditions before being transferred to constant white light. Rhythmic bioluminescence was analyzed as previously described (Knowles et al., 2008). For leaf movement analysis, seedlings were entrained for 10 d under a 12L/12D cycle and then transferred to constant white light, and the vertical position of the primary leaves was monitored and analyzed as previously described (Lu et al., 2011). Rhythm data were analyzed with BRASS (available from http://www.amilar.org) using the fast Fourier transform nonlinear least square program (Millar et al., 1995; Plautz et al., 1997).

**Measurement of Flowering Time**

Arabidopsis plants were grown on soil under either LD (16 h light/8 h dark) or SD (8 h light/16 h dark) conditions. Flowering time was scored by counting the number of days to, and number of rosette leaves at, flowering.

**RNA Extraction and RT-PCR**

One- to two-week-old seedlings were grown on Murashige and Skoog medium (Murashige and Skoog, 1962) with 1.5% agar. For the circadian
experiments, samples were collected every 4 h in continuous white light. Total RNA was isolated using the Illustra RNAspin mini kit (GE Healthcare). cDNA was synthesized from 1 µg of total RNA using the SuperScript first-strand cDNA synthesis system (Invitrogen). Quantitative (q)RT-PCR and semiquantitative RT-PCR were carried out as previously described (Lu et al., 2008). Actin2 and Actin7 were used as a noncycling reference for qRT-PCR and semiquantitative RT-PCR, respectively. Expression levels were normalized to the level of the control.

**CK2 Activity Assays**

Whole-cell extracts were prepared from 7-d-old plants. After harvesting, the plants were ground to a powder in liquid nitrogen. Fifty microliters of CK2 buffer (50 mM Tris-HCl, 10 mM MgCl2, 25 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor cocktail-EDTA [Boehringer Mannheim]) was added to approximately 100 µL of ground tissue and the mixture was briefly crushed with a small pestle. Cell debris was pelleted by centrifugation at 16,000g and discarded. Total protein concentration in the extract was determined by the Bradford assay. Quantitation of CK2 activity in the extracts was accomplished using the CK2 assay kit (Millipore, catalog no. 17-132). Fifteen micrometers of protein from the whole-cell extracts and 5 µCi [γ-32P]ATP were used for this assay. Reactions were incubated at 30°C for 10 min. For CK2 assays involving GST-CCA1 as a substrate, approximately 2 µg of GST-CCA1 bound to sepharose beads (Sugano et al., 1998) and whole-cell extracts containing 1, 5, 10, or 20 µg of total protein, were combined in reaction buffer (1× CK2 buffer, 5 mM EGTA, 0.1 mM NaVO3, 20 µM GTP, 5 µCi [γ-32P] GTP). Reactions (40 µL each) were incubated at 30°C for 10 min and then stopped by adding 2 µL of 0.5× EDTA. The beads were washed three times in 0.75 mL of wash buffer (1× CK2 buffer, 1× protease inhibitor cocktail-EDTA, 0.01% NP-40). Five microliters of 4× SDS loading buffer was added to the beads and the samples were boiled for 5 min before running on a 10% SDS gel. The gel was stained with Coomassie Brilliant Blue R-250, destained, and dried using a gel dryer. The gel was then exposed to a phosphor screen. 32P-labeled bands were quantitated using a phosphorimager and ImageJ software.

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**Plant Protein Extracts and Immunoblot Analysis**

Proteins were extracted in 1× extraction buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 10% glyc, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 50 µM MG132, and protease inhibitor cocktail [Roche]). Immunoblotting was performed as described (Lu et al., 2009) using the appropriate primary antibody (affinity-purified anti-CCA1 antibody [Wang and Tobin, 1998], anti-Actin [MP Biomedicals, Clone C4]).

Sequence data from this article can be found in the Arabidopsis Genome Initiative database using the following accession numbers: CKA1 (At1g29470), CKA2 (At4g02790), CKA3 (At1g02390), FT (At1g29920), CO (At1g51840), FLC (At2g45640), ACT2 (At2g17880), ACT7 (At5g99810), CCA1 (At1g24630), LHY (At1g10160), TOCI (At5g1380), LUX (At5g46640), GI (At1g22770), PRR7 (At5g02810), PRR9 (At2g47990), and ELF3 (At2g29590).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** The cka1/c2a2a3 triple mutations lengthen the period of expression of clock-controlled genes.

**Supplemental Table S1.** List of PCR primer sequences.

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