CikA, a Bacteriophytochrome That Resets the Cyanobacterial Circadian Clock
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The cyanobacterium *S. elongatus* PCC 7942 (1) exhibits circadian rhythms of gene expression that can be monitored using luciferase reporter genes (2). These bioluminescence rhythms persist with a period of approximately 24 hours, are temperature compensated, and their phase can be reset by light/dark transitions or by temperature cues (3). The cyanobacterial clock exhibits these characteristics of eukaryotic circadian clocks despite a lack of apparent homology between its protein components and those identified in other groups of organisms (4). For example, the complete genome sequence of *Synechocystis* sp. strain PCC 6803 is devoid of sequences similar to clock genes of *Drosophila*, such as period, timeless, Clock, and cycle, or the frequency gene of *Neurospora* (4, 5). Likewise, no homologs of the cyanobacterial *kaiA, kaiB, or kaiC* genes, essential for circadian rhythmicity (6), have been detected thus far in eukaryotes. Other cyanobacterial genes that, when mutated, affect relay of temporal information from the clock to downstream genes include a sigma factor (7) and a putative carboxylase (8). A histidine kinase, *SasA*, interacts with the *KaiC* protein and works with the oscillator either at a point of environmental input or of output transduction to all downstream genes (9). We describe here a new clock-associated gene, *ckikA*, that lies on an input pathway that supplies phase-setting information to the *S. elongatus* clock. The *ckikA* gene was identified from a Tn5 transposon insertion mutant (2) that showed subtle alteration in light-responsive regulation of a photosystem II gene, *psbAII* (10). Expression of a *psbAII::luxAB* (bacterial luciferase) fusion in the mutant was 50 to 80% of wild type under low light conditions and showed exaggerated induction on exposure to higher light intensity (11). However, a more striking *ckikA* (2, 12) phenotype was noted: the period of bioluminescence oscillation was shortened by approximately 2 hours (22.80 ± 0.45 versus 24.71 ± 0.25, n = 12), and the relative timing of peaks (phase angle) was offset by approximately 6 hours (Fig. 1A).

Reduction of both period and amplitude was observed with all reporters (Fig. 1, A to D) (e.g., periods for *kaiB::luxAB*, 22.36 ± 0.47 hours versus 25.24 ± 0.35 hours, n = 12; for *purF::luxAB*, 22.75 ± 0.24 hours versus 24.86 ± 0.33 hours, n = 12). Nonetheless, expression from the *kaiB* promoter, indicative of clock gene expression, remained robustly rhythmic with no notable alteration in phase angle (Fig. 1B). The bioluminescence rhythm from a *purF::lux* reporter (firefly luciferase) was also affected in both amplitude and period (Fig. 1C), indicating that the phenotype is not related to the substrates of bacterial luciferase and that it extends to class 2 genes (*purF* peaks at subjective dawn and is defined as class 2; the majority of gene expression patterns in the organism peak near subjective dusk and are defined as class 1 (13)). A gentamycin resistance cassette inserted in both orientations with respect to the *ckikA* open reading frame (ORF) caused phenotypes identical to those of the original Tn5 insertion mutant (Fig. 1D). Note that the *kaiA::luxAB* reporter showed an altered phase-angle phenotype; thus, in the *ckikA* genetic background, the relative phasing of *kaiA* and *kaiBC* expression is uncoupled without dramatically affecting circadian timing (Fig. 1, B and D), as was previously demonstrated for mutation of the *cpmA* gene (8).
Mutant kai alleles in a psbA1::luxAB reporter background that affect period (6) allowed us to examine the effect of cikA inactivation in both short- and long-period mutants of S. elongatus. The cikA mutation caused a very small, but reproducible, additional period shortening of the psbA1::luxAB reporter rhythm in the kaiB missense mutant B22a, and a dramatic phase-angle change (Fig. 2A). The phase-angle alteration is particularly marked in the kaiC long-period mutant C28a background (Fig. 2B), in which the C28a/cikA double mutant and wild type have a stable period length relationship throughout the run, but their bioluminescence peaks are offset by approximately 9 hours. The additive effect of the combined mutations suggests that CikA and Kai proteins perform independent, nonoverlapping functions.

Genetic complementation confirmed that inactivation of the cikA gene is responsible for the mutant phenotypes, rather than possible polar effects of the transposon insertion on nearby genes. Wild-type amplitude, period, and phase-angle properties were all restored to psbA1::luxAB bioluminescence when an ectopic copy of cikA was provided to a cikA mutant strain (Fig. 2C).

Persistence of robust circadian rhythms in the cikA genetic background indicates that the product of this gene is not essential for circadian oscillator function. The global effect on period of more than eight tested genes (14), including representatives of classes that were assigned to distinct output pathways by mutational analyses, suggests that the cikA product is not part of one of these pathways, unless it functions as does SasA in close association with the clock (9). To determine whether CikA provides environmental input to the oscillator, we tested the ability of cikA-inactivated reporter strains to reset the phase of the clock in response to a 5-hour dark pulse (15). During portions of the circadian cycle, wild-type S. elongatus responds to this stimulus by changing the phase of subsequent peaks by 10 to 12 hours after cells are returned to continuous light (Fig. 3). In contrast, cikA mutant strains show little phase resetting in this assay. These data are consistent with CikA functioning in an input pathway to the circadian oscillator.

We named the gene cikA, for circadian input kinase, on the basis of mutant phenotypes and inference from sequence analysis. The most striking features in the deduced protein sequence (16) are histidine protein kinase motifs that conform to all conserved blocks for that family (Fig. 4A, blocks H, N, D/F, and G) (17). The carboxyl terminus is similar to the receiver domains of response regulators, most notably PhoB (Fig. 4C) (18). Although other key residues of this motif are present, the invariant Asp in this family, which is the residue phosphorylated by a cognate histidine protein kinase in each case, is absent from the sequence (Fig. 4C) (16). Thus, if the CikA histidine protein kinase domain transfers a phosphoryl group to its receiver domain, another residue must become phosphorylated. Alternatively, phosphoryl transfer may not be the role of this segment of the protein; perhaps it interacts with other regulatory partners, and this contact is modulated by autophosphorylation within the H box.

The amino-terminal sequence reveals that the protein belongs to the expanding family of bacteriophytochromes (19), similar to Synechocystis sp. strain 6803 Cph1 (20), Fremyella diplosiphon RcaE (21), Deinococcus radiodurans BphP (22), and Arabidopsis thaliana PhyE (23) (Fig. 4B). This raises the possibility that CikA is a photoreceptor. However, unlike other known phytochromes and bacteriophytochromes, CikA lacks the conserved Cys residue expected as a bilin ligand for phytochromes (24, 25). It also lacks the His residue reported to be the bilin ligand for D. radiodurans BphP, which corresponds to His 323 in the PhyE sequence (Fig. 4B) (22). This suggests several possi-

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**Fig. 1.** Circadian phenotypes of reporter strains in which cikA is inactivated by transposon Tn5 insertion. Bioluminescence (counts per second) is shown from (A) translational luxAB fusion to psbA1 (10); (B) transcriptional luxAB fusion to the promoter of kaiB (6); (C) translational fusion of a firefly luciferase gene (lux) to purF (33); and (D) translational luxAB fusion to kaiA (6). In (A), (C), and (D), black bars on the abscissa indicate dark incubation periods; otherwise, samples were kept in continuous light (onset = time 0). In (B), samples were subjected to one 12-hour dark incubation before release into continuous light. Green, wild type; blue, cikA insertion mutant. (D) Blue diamonds, Tn5 insertion mutant; blue circles and squares, two orientations of inactivating genta-mycin resistance cassettes.

**Fig. 2.** (A and B) cikA disruption in period mutants of Synechococcus. Wild-type (green; period 25.11 ± 0.46 hours, n = 12) and period mutant kai backgrounds (rose) carry a psbA1::luxAB fusion (6, 34). (A) Rose, period mutant B22a (21.34 ± 0.10 hours, n = 5); blue, B22a/cikA (20.92 ± 0.34 hours, n = 5). (B) Rose, period mutant C28a, (26.36 ± 0.83 hours, n = 5); blue, C28a/cikA (24.34 ± 0.13, n = 5). (C) Genetic complementation of the cikA phenotype. Ectopic insertion of a copy of cikA (red) restored the period, amplitude, and phasing phenotypes of a cikA disruption mutant (blue) to match those of wild type (green), as measured by a psbA1::luxAB reporter. Ectopic insertion of the extra copy of cikA into a wild-type background (black). Axes labeled as in Fig. 1.
Fig. 3. Phase-resetting of the psbA-luxAB bioluminescence rhythm in wild-type (diamonds), cikA (squares), and complemented cikA (triangles) genetic backgrounds in response to a 5-hour dark pulse. At the indicated circadian time on the abscissa, samples received 5 hours of dark incubation, then were returned to continuous light for monitoring of the circadian rhythm (15). The ordinate for each data point indicates the offset of the phase of peaks after the treatment, relative to a control not pulsed with darkness: phase advance (positive values) or phase delay (negative values). To accommodate differences in circadian period between strains, actual time was converted to circadian time (one circadian hour = free running period × 24\(^{-1}\)).

Fig. 4. (A) Graphic representation of the 754-amino acid CikA protein, indicating relative size and distribution of identifiable motifs: chromophore binding domain of phytochromes (CB); H, N, D/F, and G boxes of histidine protein kinases; and a receiver domain of response regulators (RR). (B) Comparison of chromophore binding domains of CikA, PhyE from Arabidopsis thaliana (GenBank accession no. X76610), Cph1 (Kazusa DNA Institute Cyanobase ORF slr0473), and slr1699 (Kazusa DNA Institute Cyanobase ORF slr1699) from Synechocystis sp. strain PCC 6803, and RcaE from Fremyella diplosiphon (GenBank accession no. US9741). Black diamond, PhyE residue 322 bilin chromophore ligand. Residues conserved in all sequences, white letters on black; in four out of five, white on grey; in three out of five, black on gray. Numbers at the beginning of each line indicate position in the respective protein sequence. Asterisks mark each tenth residue in alignment. (C) Comparison of receiver domains of CikA, slr1699 from Synechocystis sp. strain PCC 6803, and PhoB from Escherichia coli (GenBank accession no. P08402). Black diamond, residue expected to be Asp in response regulator receiver domains. Black background, identical residues; grey background, chemically similar residues. For alignments (B and C), we used a ClustalW 1.8 alignment tool accessed through the BCM Search Launcher (35). Alignment in (B) was modified by hand on the basis of information from 54 phytochrome-like sequences with assistance from C. Lagarias (36).

Patterns for CikA structure and function: it does not bind a bilin chromophore, it binds a chromophore (bilin or another cofactor) non-covalently, or it binds a chromophore by a novel arrangement.

The similarity of CikA to phytochromes provides the first potential evolutionary parallel between cyanobacterial and eukaryotic circadian systems. Phytochromes play several distinct roles in relaying light information to the circadian clocks of plants (26). Although the white collar proteins of Neurospora, important for light-dependent processes and for circadian clock function (27), bear similarity to phytochromes, the correspondence is through shared PAS domains. No direct link can be drawn between the white collar proteins and CikA, which lacks a PAS domain and resembles a different part of the phytochrome sequence—

the chromophore binding domain.

A subsequent direct screen for transposon mutants that affect phase resetting has identified five independent cikA mutants, and no other loci, as causing clear resetting phenotypes (28). This further supports a key role for CikA in providing environmental input to reset the cyanobacterial circadian clock.

References and Notes

1. This strain has been reported without a specific name as Synechococcus sp. strain PCC 7942 (Pasture Culture Collection accession no. 7942). The strain PCC 6301 has been proposed as the living neotype of S. elongatus (29, 30); a pending update to Berger’s Manual of Determinative Bacteriology will reflect this nomenclature. PCC 7942 is very closely related to PCC 6301 (31, 32) and, thus, can be assigned to S. elongatus.


11. O. Schmitz et al., data not shown.


14. O. Schmitz et al., data not shown.

15. The experimental design for Fig. 3 was modified from phase-response curve protocols developed by K. Okamoto, C. Inoue, and T. Kondo (unpublished data). Supplemental information is available to Science Online subscribers www.sciencemag.org/data/1051545.txt.

16. Nucleotide and deduced amino acid sequences for cikA are entered in the GenBank database (accession no. AF258464). Absence of the Cys residue corresponding to PhyE 322 was confirmed by direct sequencing from a polymerase chain reaction amplification product of PCC 7942 chromosomal DNA in the chromophore binding domain. Absence of the conserved Asp in the receiver domain is supported by independent database entry of sequence flanking the S. elongatus PCC 6301 gsa gene, the locus immediately downstream of cikA (GenBank accession no. AF038423).


28. O. Schmitz et al., data not shown.


Cloning of the Arabidopsis Clock Gene TOC1, an Autoregulatory Response Regulator Homolog

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The toc1 mutation causes shortened circadian rhythms in light-grown Arabidopsis plants. Here, we report the same toc1 effect in the absence of light input to the clock. We also show that TOC1 controls photoperiodic flowering response through clock function. The TOC1 gene was isolated and found to encode a nuclear protein containing an atypical response regulator receiver domain and two motifs that suggest a role in transcriptional regulation: a basic motif conserved within the CONSTANS family of transcription factors and an acidic domain. TOC1 is itself circadianly regulated and participates in a feedback loop to control its own expression.

The endogenous circadian clock enables organisms to anticipate and adapt to daily variations in the environment and to temporally coordinate internal processes. In animals, fungi, and bacteria, genetic screens for altered circadian rhythms have revealed molecular clock components. The generally conserved core mechanism consists of autoregulatory transcriptional loops in which positive factors act on genes encoding negative factors that in turn feed back to block their own expression (1). Although plant models have proven valuable for understanding circadian input and output pathways, our understanding of processes at the core of the plant circadian system is lacking.

We therefore executed a screen for rhythm mutants in Arabidopsis, from which we identified the toc1 (timing of CAB expression) mutant (2). The defining phenotype is a shortened period of luciferase-reported CAB gene expression (~21 hours, versus ~24.5 hours in the wild type) under constant light conditions (LL). All clock phenotypes tested are similarly affected by the toc1-1 mutation, which is semidominant, as are mutant alleles of diverse clock genes (1–3). Moreover, the effects of the toc1-1 mutation are specific to the clock system, with no defects seen, for instance, in clock-independent light responses (3). This is noteworthy because disruption of photoreceptors and phototransduction components that participate in clock entrainment can alter period in LL (4, 5). However, perturbations of these components produce specific, differential effects depending on the quality and quantity of light, whereas the toc1-1 effect is essentially the same in all light conditions (3). To further address this issue, we assayed the bioluminescence rhythm of toc1-1 and wild-type seedlings during extended dark incubation (DD) using a new reporter, cerc2::luc. CCR2 (COLD–CIRCADIAN RHYTHM–RNA-BINDING) 2 is a clock-controlled gene whose LL expression rhythm is shortened by the toc1-1 mutation (6). The cerc2::luc reporter (including a luciferase gene fusion) reveals that toc1-1 has a similar effect on the period of gene expression in DD (Fig. 1), consistent with a role for TOC1 outside of light input pathway(s) to the clock.

Mutation of TOC1 also affects photoperiodic regulation of floral induction. Wild-type Arabidopsis flowers earlier in long days [16 hours light, 8 hours dark (16:8 LD)] than in short days (8:16 LD), but this differential response is greatly reduced in toc1-1 (3). This phenotype is likely the result of clock-based misinterpretation of photoperiodic information in toc1-1 rather than direct effects of toc1-1 on floral induction pathways. To test this possibility, we measured the transition to flowering of toc1-1 lines grown in LD cycles of 21 hours total duration, where the environmental period (T) more closely matched the period of the endogenous clock (τ) (Fig. 2). Correct photoperiodic response was restored in toc1-1 plants grown in this regime, where toc1-1 plants flowered much later in short days (7:14 LD) than in long days (14:7 LD). The toc1-1 flowering defect therefore can be fully explained by its circadian defect. The cause is not simply incorrect measurement of light or dark intervals: Mutant plants given 7 hours light in a 24-hour period (7:17 LD) also flowered early (Fig. 2). These data, combined with results of experiments measuring gene expression in toc1-1 and wild-type plants entrained to altered T cycles (3), also suggest a possible mechanism underlying this defect: incorrect modulation of phase and/or waveform of clock-controlled regulatory factors when τ varies greatly from T.

To further investigate its role in the Arabidopsis circadian system, we isolated the TOC1 gene. Genetic mapping delimited

![Fig. 1. Bioluminescence rhythms from toc1-1 and wild-type seedlings in constant darkness (DD). cerc2::luc transgenic seedlings (7) were grown in 12:12 LD for 8 days before transfer to DD. Bioluminescence was recorded at the indicated times (7). Traces represent averages of 21 to 23 seedlings from each line. Period estimates (variance-weighted means ± variance-weighted SD) for each line were calculated as described (26, 27): wild type = 27.5 ± 1.16 hours, toc1-1 = 22.3 ± 0.39 hours.](https://www.sciencemag.org/content/full/289/5473/1844/F1.large.jpg)