Supporting Information Appendix for Phase-resetting mechanism of the circadian clock in *Chlamydomonas reinhardtii*

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SI Materials and Methods

Strains and media

The CBR mating type minus (mt^-) strain of *C. reinhardtii* (1) and its sibling, CBR mt^+, were used as WT reporter strains that carry the *tufA* promoter-*lucCP* reporter gene in their chloroplast genomes (2). We used circadian rhythm mutants: *roc15*, *roc69*, *roc78*, and *roc94* (an allelic mutant of Mut-9 [3]), *roc108*, and *roc114* (1). These mutants carry the *tufA* promoter-*lucCP* reporter. Since *roc114* and *roc108* are allelic mutants of the clock gene *ROC114*, we hereafter referred to these mutants as *roc114-1* and *roc114-2*, respectively.

A reporterless WT strain was generated for this study by crossing the CBR mt^- strain to the 21gr mt^+ strain (from the Chlamydomonas Resource Center [http://chlamycollection.org/]). A reporterless progeny was backcrossed for 4 generations to CBR mt^- to recover the CBR genetic background. The chloroplast genome carrying the luciferase reporter was not inherited from the mt^- parent. We termed the progeny the BR strain (mt^+ and mt^-) and used it as a reporterless WT strain.

For culture of *C. reinhardtii*, we used Tris-acetate-phosphate (TAP) (4) and high-salt media (5). The light response of ROC15-LUC was essentially the same in both media.

Generation of luciferase reporter strains

Clock protein-luciferase fusion genes were generated as follows. The stop codon of the clock gene on the genomic DNA fragments (roc15KK, roc40HH, roc66ES, and roc75BB) (1) cloned in the pSI103PL vector was replaced with a *Cla*I or *Fse*I restriction site by PCR-based mutagenesis. A coding sequence for the codon-adapted firefly luciferase (*LUCnc*; GenBank/EMBL/DDBJ accession no. AB762768) was generated by artificial gene synthesis (Hokkaido System Science, Sapporo, Japan) and cloned into the pCR2.1TOPO vector (Life Technologies), yielding the pCR2.1TOPO/LUCnc construct. *Cla*I and *Fse*I sites and a sequence for a flexible glycine-serine (GS) linker were attached to the *LUCnc* by PCR. A *Cla*I or *Fse*I (partial digestion) fragment encoding the GS linker-LUCnc was inserted into the *Cla*I or *Fse*I sites replaced from the stop codon of the clock genes (Fig. S1*A*). *Pac*I fragments containing the luciferase-inserted clock gene fragment and the paromomycin-resistance marker were used for transformation of the BR mt^+ strain (Fig. S1*A*). Transformation was carried out as described previously with 300 ng of transforming DNA (1). To enhance the transformation rate, cells were pretreated with gametolysin (6, 7).

Bioluminescence monitoring

Luciferin (Biosynth, Staad, Switzerland) was added to the media at a final concentration of 200 μM, and bioluminescence was monitored at 17°C. Bioluminescence of cultures in white 96-well

plates was measured using the apparatus described by Okamoto et al. (8), and cultures in black 24-well plates (VisiPlate-24; PerkinElmer) were measured using a newly developed highly sensitive monitoring apparatus (model CL24-W; Churitsu Electric Corp., Nagoya, Japan). To decrease the delayed light emission of chlorophyll, samples during light periods were subjected to darkness for 210 sec just before bioluminescence measurement at each time point. For monitoring by the highly sensitive apparatus (CL24-W), background luminescence levels of cultures without luciferin were subtracted from the bioluminescence levels of luciferin-added cultures.

Light sources

We used fluorescence tubes (FLR 40S W/M; Panasonic) for white illumination and LEDs for blue (470 nm with a full-width at half maximum [FWHM] of 27 nm), green (525 nm [FWHM of 34 nm]), and red (660 nm [FWHM of 24 nm]) illuminations (ISL-150x150-BB, ISL-150x150-GG, and ISL-150x150-RR, respectively; CCS, Kyoto, Japan). We also used additional LEDs for blue (470 nm [FWHM of 25 nm]) and red (660 nm [FWHM of 18 nm]) illuminations (MIL-B18 and MIL-R18, respectively; Panasonic). Fluence rates were measured at the surface level of plates or flasks by using a light meter (Model LI-250; LI-COR, NE, USA) attached to a quantum probe (LI-190; LI-COR).

Expression analyses

Northern blot analysis was performed as described previously (2). For western blot analysis, cells were harvested by centrifugation, resuspended in a lysis buffer (50 mM Tris pH 8.0, 2% SDS, 10 mM EDTA, 1 μL/mL protease inhibitor cocktail [Sigma]; plus NaF [5 mM] and Na_3VO_4 [1 mM] as necessary), and incubated at room temperature for 30 min in the dark. Insoluble debris was pelleted by centrifugation (15 min, 20630 × *g*, 4°C), and the supernatant was used as the cell lysate. The protein concentration was determined by an improved Bradford method (BradfordUltra; Expedeon, Cambridge, UK). The cell lysate was separated by SDS-PAGE and blotted onto a polyvinyl difluoride (PVDF) membrane (Immobilon-P; Merck Millipore). For detection, mouse monoclonal anti-HA antibody (1:1000; COVANCE, clone 16B12) and horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:25000; GE Healthcare) were used as primary and secondary antibodies, respectively.

Phosphatase treatment

Cells were harvested by centrifugation, resuspended in a phosphatase buffer (10 mM Tris pH 7.5, 150 mM NaCl, 0.1 % IGEPAL, 1 mM phenylmethylsulfonyl fluoride, 10 μL/mL protease inhibitor cocktail [Sigma], 1 mM dithiothreitol), and sonicated for 30 sec on an ice bath using a sonicator (Sonic Vibracell VC750, Sonics & Materials) with a 3 mm tapered micro tip at 40 %

amplitude. Insoluble debris was pelleted by centrifugation (10 min, 20630 × *g*, 4°C), and the supernatant was used as the cell extract. For phosphatase treatment, λPPase (New England Biolabs) and manganese chloride were added to the extract at the final concentration of 20000 U/ml and 1 mM, respectively, and the reaction was incubated at 30°C for 30 min. Duplicate reaction without λPPase was included as negative control.

Immunocytochemistry

Cells were harvested by centrifugation and fixed by suspending in phasphate-buffered saline (PBS) containing 1 % paraformaldehyde at 4°C for one hour. The fixed cells were seeded to poly-L-lysine coated coverslips (Asahi Techno Glass Corp., Tokyo, Japan), and permeabilized with methanol twice each for 10 min at -20°C. Cells were rehydrated with PBS. Thereafter, cells were treated with a blocking buffer (5 % bovine serum albumin, 1 % cold water fish skin gelatin, 10 % goat serum in PBS), primary antibodies at 4°C for overnight, and secondary antibodies at room temperature for 2 h. Between each treatment, the coverslips were washed five times in PBS. As primary antibodies, we used mouse monoclonal anti-HA antibody (1:1000; COVANCE, clone 16B12), rat monoclonal anti-HA antibody (1:1000; Roche, clone 3F10), mouse monoclonal anti-NPC antibody (1:0000; COVANCE, clone MAb414), and rabbit polyclonal anti-TAP tag antibody (1:250; GenScript). As secondary antibodies, we used AlexaFluor488-conjugated $F(ab')_2$ fragment of goat anti-rabbit IgG (Life Technologies), AlexaFluor488-conjugated goat anti-rat IgG (Life Technologies), and AlexaFluor546-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG (Life Technologies). All antibodies were diluted in the blocking buffer. Fluorescence was observed with a laser-scanning confocal fluorescence microscope (LSM5Pascal, Carl Zeiss) and a fluorescence microscope (IX70, Olympus) equipped with a CCD camera (DS-5M, Nikon).

Inhibitors

Inhibitors used in this study and the concentrations of stock solutions were follows: cycloheximide (100 mg/mL in DMSO; Sigma-Aldrich), 6-DMAP (60 mM in H_2O; Merck Millipore), MG-132 (10 mM in DMSO; Merck Millipore), lactacystin (10 mM in DMSO; Merck Millipore), proteasome inhibitor I (10 mM in DMSO; Merck Millipore), epoxomicin (10 mM in DMSO; Merck Millipore), and DCMU (100 mM in EtOH; Wako, Osaka, Japan). The final concentrations are shown in the figure legends.

SI Figures

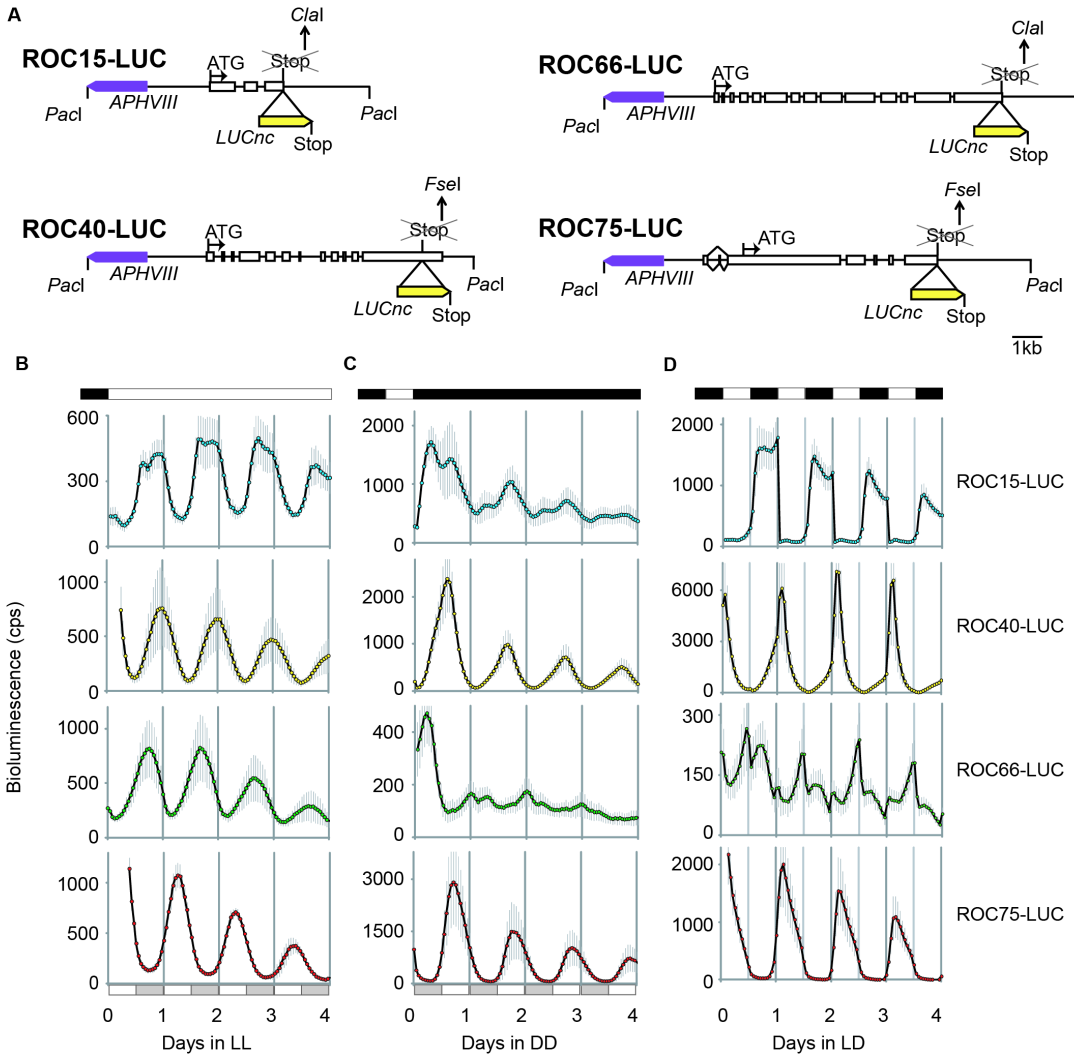

Fig. S1. Bioluminescence monitoring of clock protein reporter strains. (*A*) A schematic view of the reporter genes. The luciferase fragment was inserted into the genomic DNA fragments of clock gene loci. White boxes represent exons of clock genes. Note that the 5′- and 3′- ends of noncoding regions have not yet been identified. *APHVIII* is a selectable marker gene that confers paromomycin-resistance on *C. reinhardtii* (9). (*B–D*) Bioluminescence traces of clock protein reporter strains under LL, DD, and LD conditions. Asynchronous spot cultures of *C. reinhardtii* cells were prepared in white 96-well plates as described previously (1) and were subjected to the light conditions indicated on the top of each graph. White and black bars represent light (2 μmol m^{-2} s^{-1}) and darkness, respectively. The bottom white and gray bars represent the subjective day and subjective night, respectively. Each point represents the mean ± SD of bioluminescence counts from 6 independent cultures. Similar results were obtained in 2-5 independent experiments and in experiments with another transgenic line (*B-D*). White light was used in all experiments in Fig. S1.

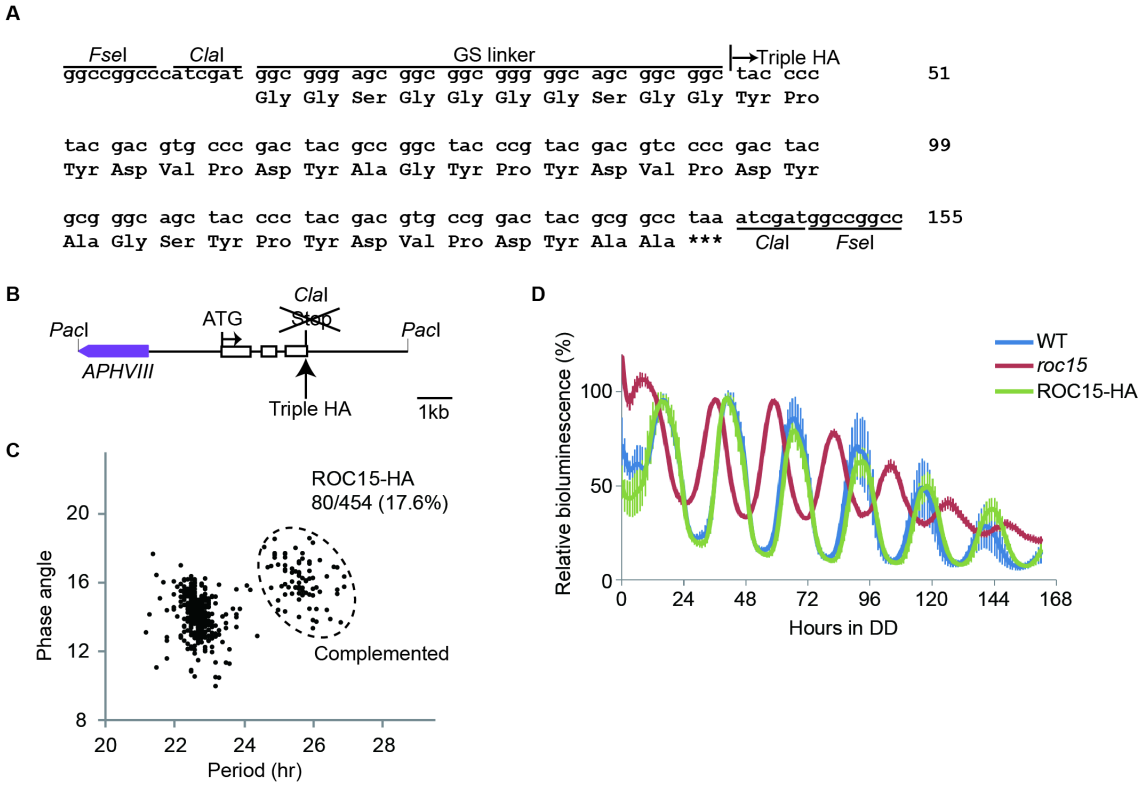

Fig. S2. Generation of the ROC15-HA strain. (*A*) Sequence of a codon-adapted triple HA tag for the *C. reinhardtii* nuclear genome. (*B*) A schematic view of the *ROC15-HA* gene. (*C*) Complementation of the *roc15* short-period and advanced-phase-angle phenotypes by the *ROC15-HA* gene. Mutant *roc15* cells were transformed with *ROC15-HA*, and bioluminescence rhythms of the chloroplast luciferase reporter (2) of spot cultures of the transformants were monitored in DD. A scatter plot of the period length and phase angle of each transformant is shown. The period and phase angle were calculated with the Rhythm-Analyzing Program (RAP) (10). The rate of complementation is indicated in the graph. A total of 17.6% of transformants showed normal circadian rhythms, indicating complementation of *roc15* by *ROC15-HA*. (*D*) Representative bioluminescence traces of WT, *roc15*, and a complemented transformant (ROC15-HA). The peak values of each trace after 24 h in DD were normalized to 100. Each point represents the mean ± SD of the normalized bioluminescence traces from 6 independent cultures.

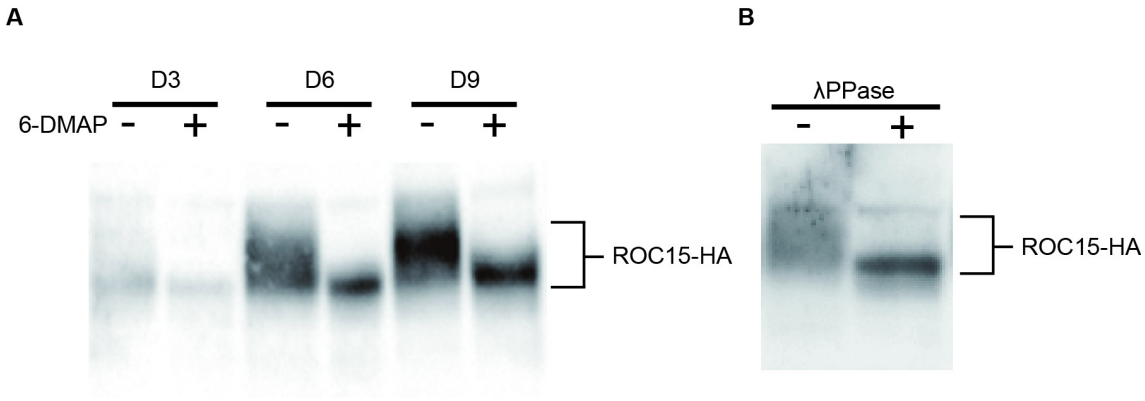

Fig. S3. Phosphorylation of ROC15-HA protein in the dark. Cells were prepared as described in Fig. 1*B*. (*A*) Representative result of Western blot analysis of 6-DMAP treated cells. 6-DMAP was added to the cultures at D0 (final conc., 3 mM), and samples were harvested at the time points indicated. (*B*) Representative result of Western blot analysis of λPPase treated cell extracts. Samples were harvested at D9, and protein extracts were treated with λPPase.

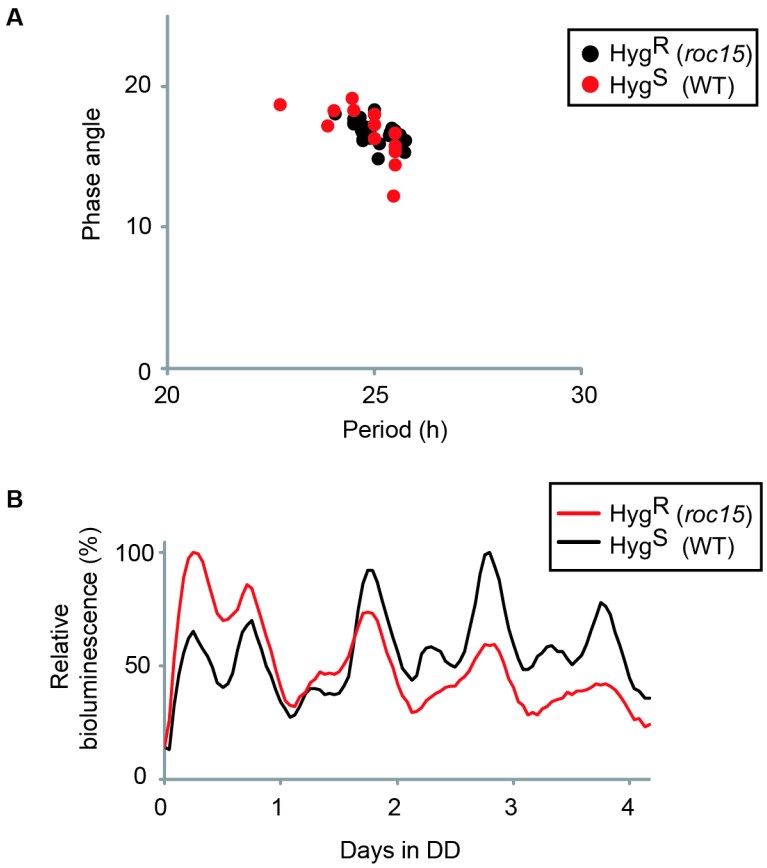

Fig. S4. Complementation of *roc15* mutant phenotypes by *ROC15-LUC*. The ROC15-LUC strain (mt^+) was crossed with the *roc15* mutant (mt^-), and spot cultures of progeny were prepared. Bioluminescence rhythms of the spot cultures derived from ROC15-LUC itself were monitored in DD. Since *roc15* was generated by insertional mutagenesis of the *Aph7″* hygromycin-resistance gene, the hygromycin-resistance is genetically linked to the *roc15* mutation (1, 11). (*A*) A scatter plot of the period length and phase angle of hygromycin-resistant (Hyg^R) and -sensitive (Hyg^S) progeny. All progeny of Hyg^R (*roc15* background) showed almost the same phase and period as the Hyg^S (WT background) progeny in the bioluminescence rhythm of ROC15-LUC, indicating complementation of *roc15* by *ROC15-LUC*. (*B*) Representative traces of bioluminescence rhythms. The peak values of each trace were normalized to 100.

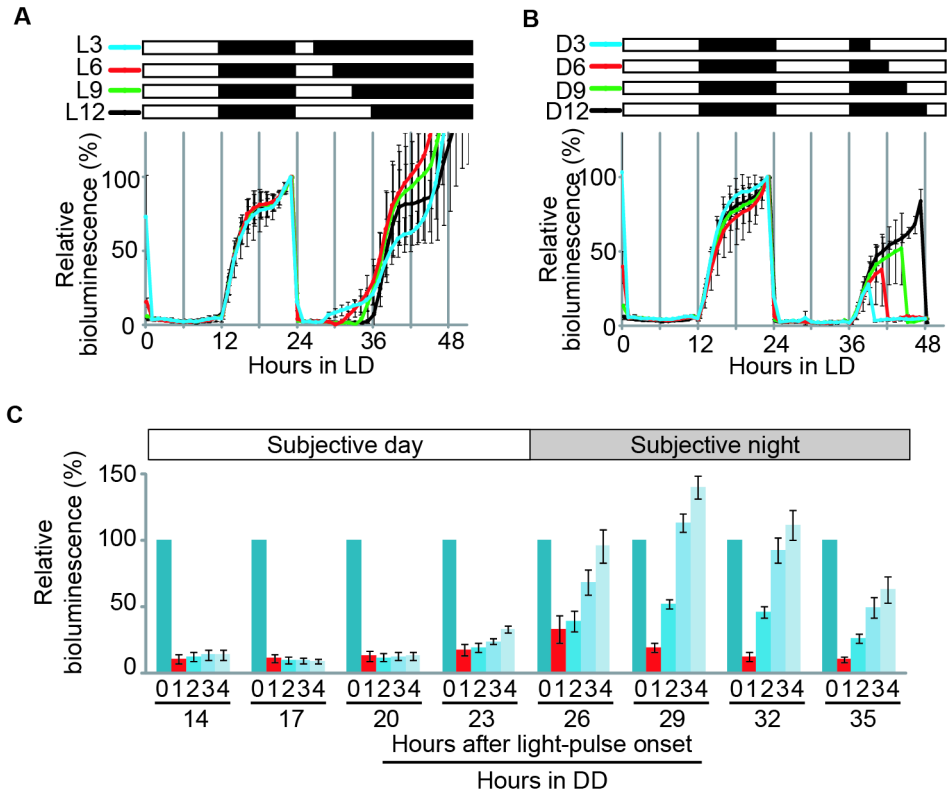

Fig. S5. Time-of-day dependence of ROC15-LUC responses. LD-entrained spot cultures were exposed to premature darkness (*A*) or light (*B*). The maximum values on the first day in the graph were normalized to 100, and data shown are the mean ± SD of the normalized traces from 6 independent cultures. (*C*) Circadian profile of the light-induced decrease in ROC15-LUC bioluminescence. Spot cultures under DD conditions were exposed to a 5-min light pulse (85 µmol m^{-2} s^{-1}) at different times of the day. Data shown are the mean ± SD of values relative to those before light pulses from 8–10 independent cultures. Similar results were obtained in 2-5 independent experiments and in experiments with another transgenic line (*A*-*C*). White light was used in all experiments in Fig. S5.

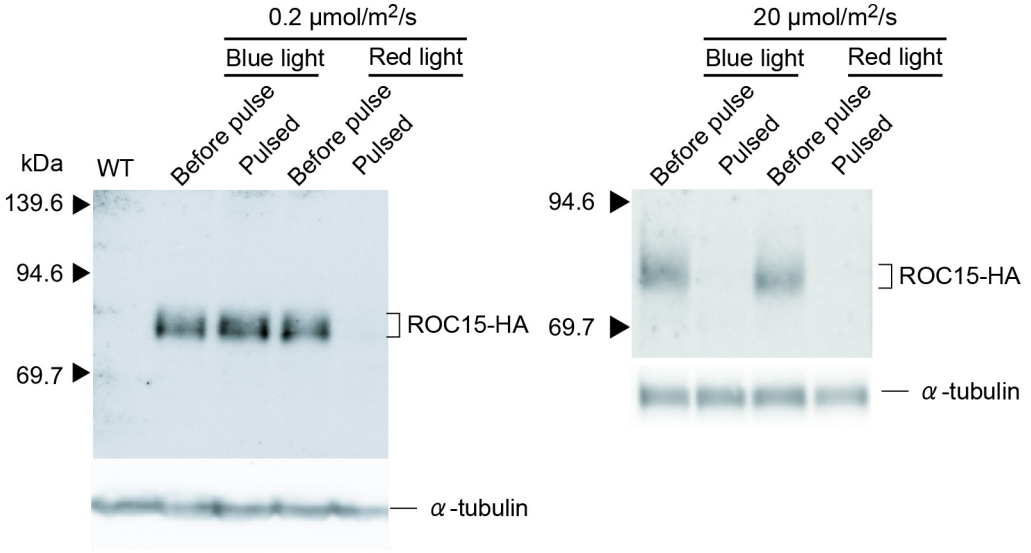

Fig. S6. The effects of blue and red light pulses on ROC15-HA. Asynchronous TAP-cultures of *ROC15-HA* without luciferin were exposed to darkness for 9 h, and cells were then exposed to blue and red light pulses (0.2 or 20 µmol m^{-2} s^{-1}, 5 min). Cells were harvested just before the light pulse and at 35 min after the onset of the light pulse.

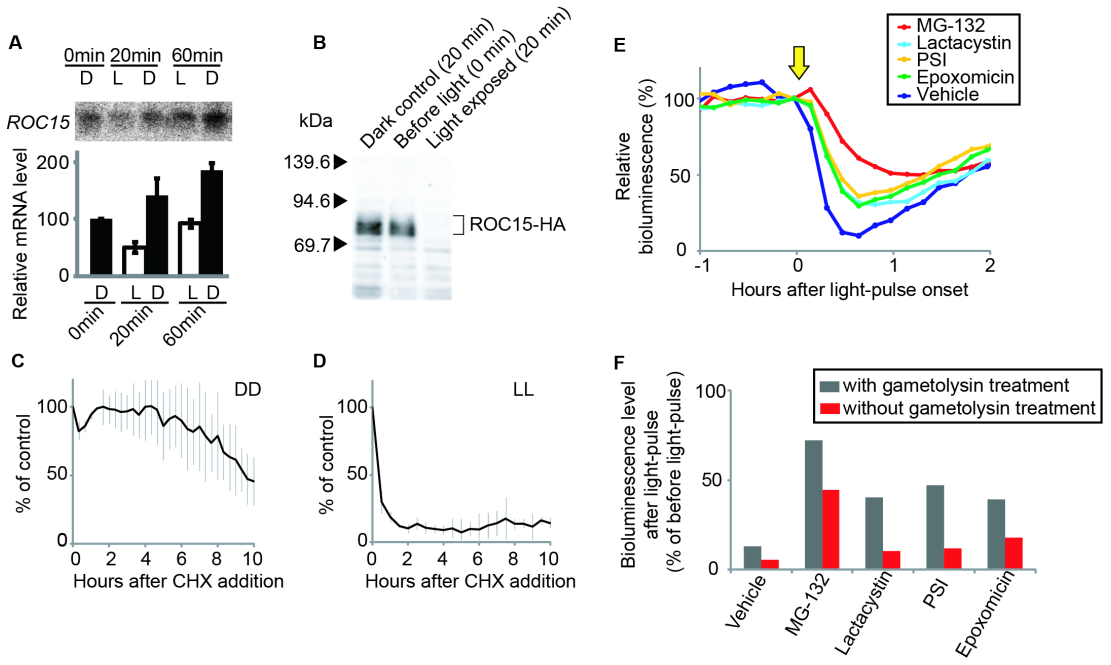

Fig. S7. Expression analyses and inhibitor experiments. (*A*) Northern blot analysis. Asynchronous TAP-cultures of WT alga were maintained to darkness for 9 h and then exposed to light. Samples were harvested at 0 (before light), 20, and 60 min (L: light-exposed [20 µmol m^{-2} s^{-1}], D: dark control). The graph shows the mean ± SD of 3 independent cultures. Data are expressed as percentages relative to the 0-min level. (*B*) Western blot analysis. Cultures of ROC15-HA strain were prepared and exposed to light as described in *A*. Samples were harvested at 0 (before light) and 20 min. (*C*) Stability of ROC15-LUC bioluminescence in darkness. CHX was added to ROC15-LUC cultures at 8 h after transfer to darkness at a final concentration of 10 µg/mL. Data shown are the means ± SD (n = 3) of values relative to the vehicle-treated cells. (*D*) Stability of ROC15-LUC bioluminescence in the light. CHX was added (final conc., 10 µg/mL.) at the mid subjective night (20 h in LL). Data are shown as in *C* (n = 4). (*E*) Effects of various proteasome inhibitors. Cells were treated with inhibitors (MG-132 [100 µM], lactacystin [50 µM], proteasome inhibitor I (PSI) [50 µM], or epoxomicin [50 µM]) as described in Fig. 3, and exposed to a light pulse (2 µmol m^{-2} s^{-1}, 0.5 min). The bioluminescence level just before the light pulse was normalized to 100. Similar results were obtained in 2 independent experiments and in experiments with another transgenic ROC15-LUC reporter line. (*F*) Effects of proteasome inhibitors on cells with and without permeabilization. Cells were prepared as described in Fig. 3, and exposed to a light pulse (2 µmol m^{-2} s^{-1}, 0.5 min). Bars represent relative bioluminescence levels at 30 min after the light pulse. Cells without gametolysin treatment showed relatively low bioluminescence level compared to cells with gametolysin treatment, indicating weaker effects of the drugs on ROC15 decline in unpermeabilized cells. White light was used in all experiments in Fig. S7.

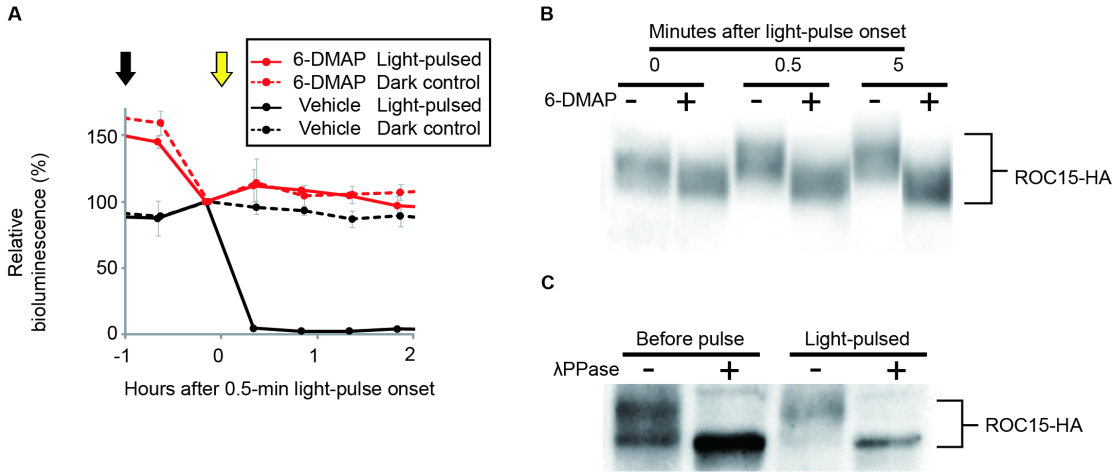

Fig. S8. Phosphorylation of ROC15 protein by light. (*A*) Effect of kinase inhibitor on the decrease in ROC15-LUC bioluminescence. The bioluminescence of ROC15-LUC was induced by exposure to darkness for 9 h followed by exposure to a white light pulse (2 µmol m^{-2} s^{-1}, yellow arrows). 6-DMAP was added to the cultures (final conc., 3 mM) 1 h before the light pulse (black arrows). The values just prior to the light pulse were normalized to 100. Each point indicates the mean ± SD of the normalized traces from 3 independent cultures. Similar results were obtained in 5 independent experiments and in experiments with another transgenic line. (*B*) Representative result of Western blot analysis of 6-DMAP treated cells. Cells were prepared as described in Fig. 1*B*, and 6-DMAP was added to cultures at D8. A white light pulse (2 µmol m^{-2} s^{-1}) was given to the cultures at D9 and samples were harvested at the time points indicated. (*C*) Representative result of Western blot analysis of λPPase treated protein extracts. Samples were harvested 5-min after the light pulse, and protein extracts were treated with λPPase.

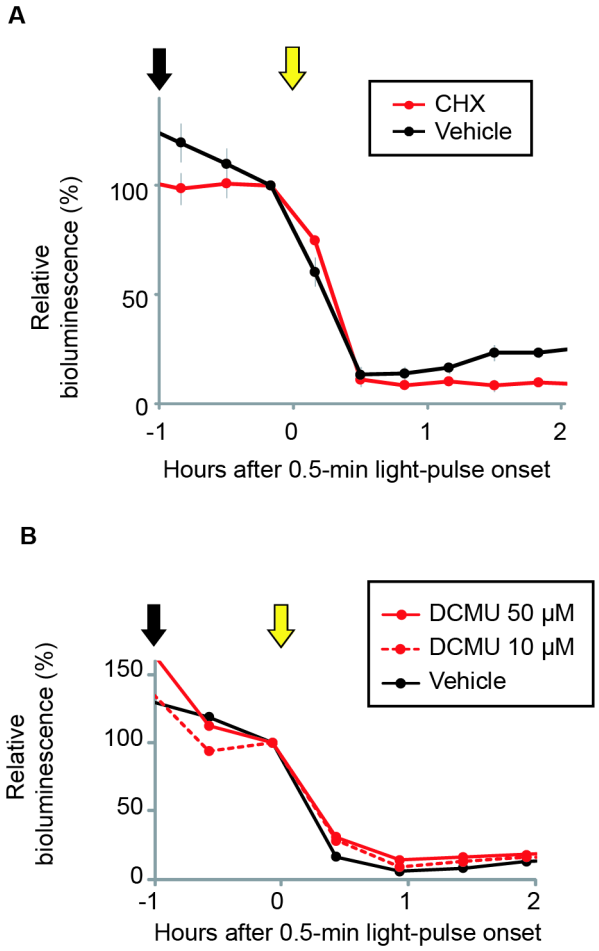

Fig. S9. Effects of translation (*A*) and photosynthesis (*B*) inhibitors on the decrease in ROC15-LUC bioluminescence. The bioluminescence of ROC15-LUC was induced by exposure to darkness for 9 h followed by exposure to a white light pulse (2 µmol m^{-2} s^{-1}, yellow arrows). The inhibitors CHX (final conc., 10 µg/ml) and DCMU (final conc., 10 and 50 µM) were added to the cultures 1 h before the light pulse (black arrows). The values just prior to light pulses were normalized to 100. Each point indicates the mean ± SD of the normalized traces from 3 independent cultures (*A* and *B*). Similar results were obtained in 2–5 independent experiments and in experiments with another transgenic line (*A* and *B*).

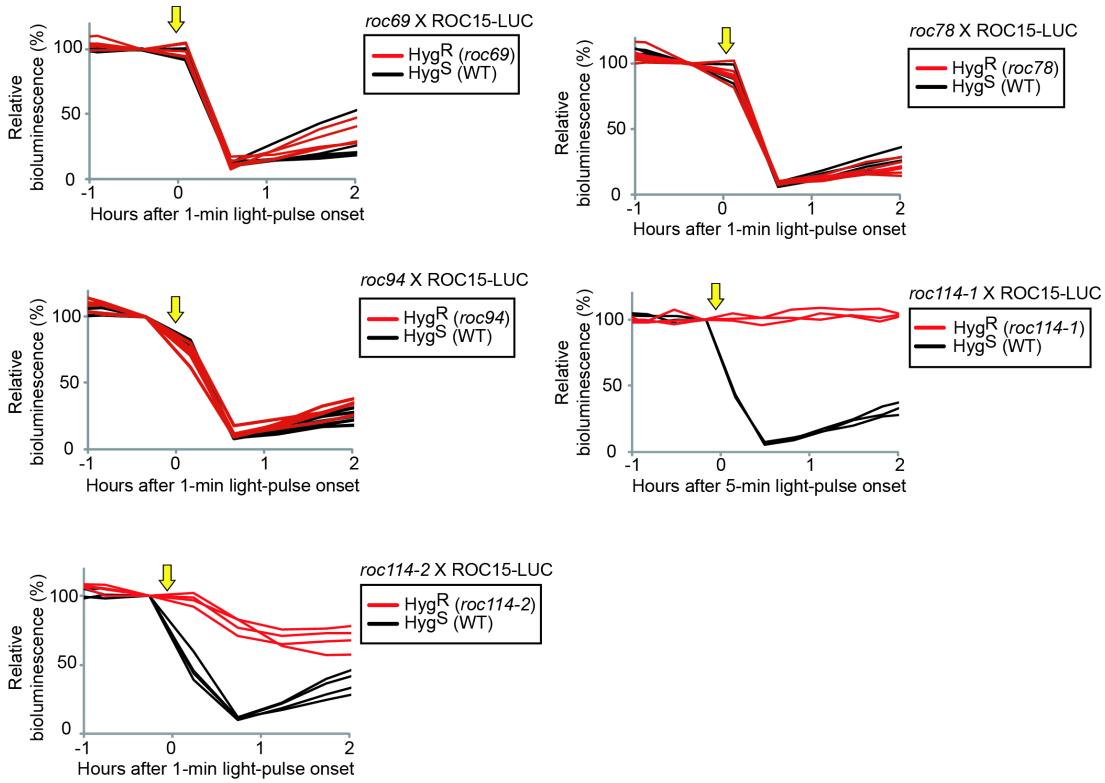

Fig. S10. The light response of the ROC15-LUC reporter in mutant strains of some kinase genes and an F-box protein gene. The ROC15-LUC reporter strain was crossed with mutants (*roc69*, *roc78*, *roc94* [Mut-9], *roc114-1,* and *roc114-2*) generated by insertional mutagenesis of the hygromycin-resistance gene (1). Their mutant phenotypes are genetically linked to their hygromycin resistance (1). Asynchronous TAP cultures of progenies were transferred to white 96-well plates, subjected to darkness for 9 h, and exposed to a white light pulse (2 µmol m^{-2} s^{-1}, arrows). Representative traces of hygromycin-resistant (Hyg^R) and -sensitive (Hyg^S) progenies are indicated. The values just prior to the light-pulse were normalized to 100.

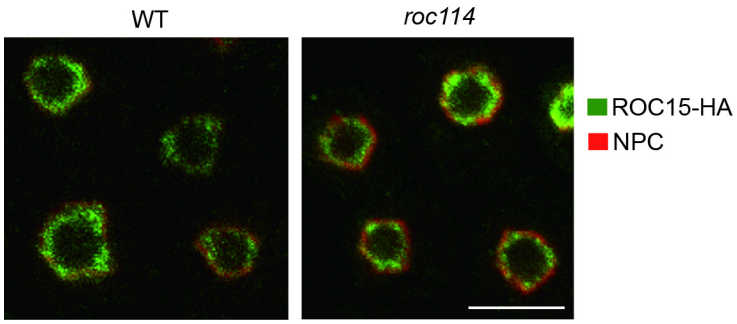

Fig. S11. Subcellular localization of ROC15. Asynchronous TAP cultures of ROC15-HA strain were exposed to darkness for 9 h. Cells were harvested and subjected to immunocytochemistry. Representative confocal images are shown. Green and red represent ROC15-HA and NPC, respectively. Scale bar represents 5 μm.

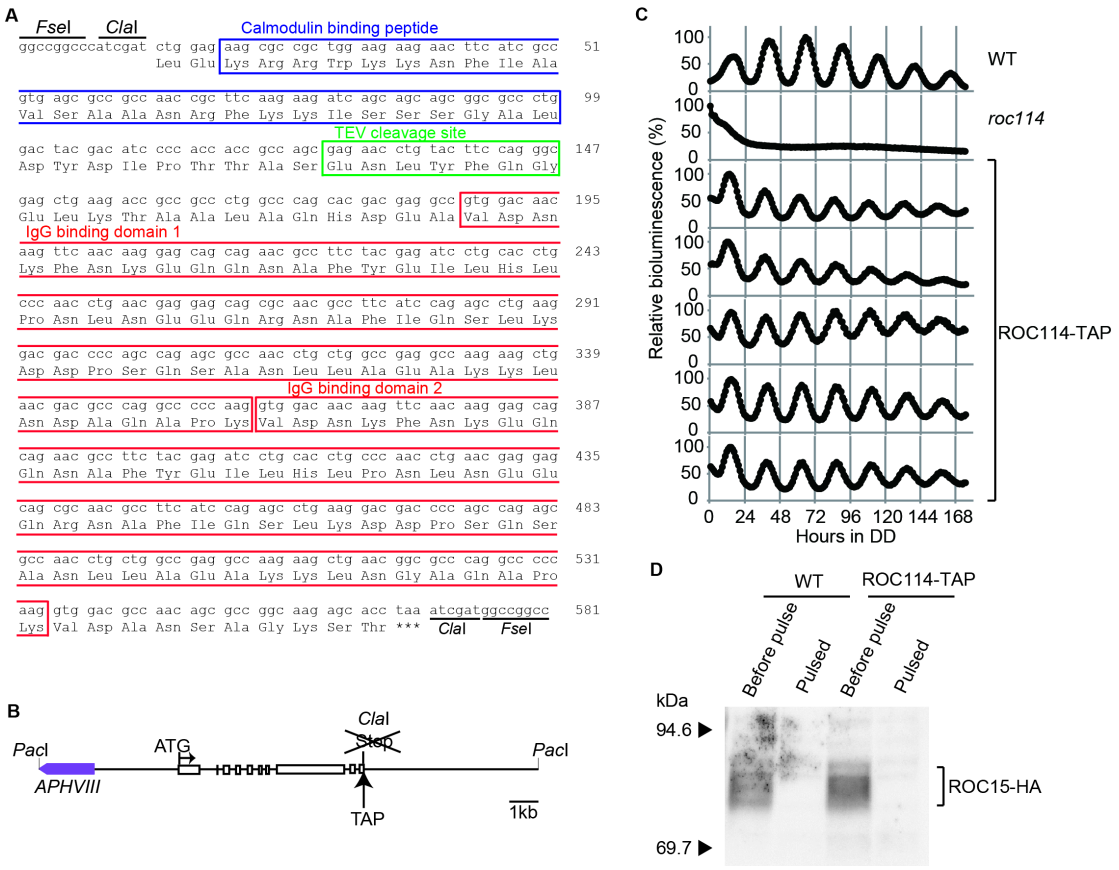

Fig. S12. Generation of the ROC114-TAP strain. (*A*) Sequence of a codon-adapted TAP tag for the *C. reinhardtii* nuclear genome. (*B*) A schematic view of the *ROC114-TAP* gene. (*C*) Complementation of the *roc114-1* arrhythmic phenotype by the *ROC114-TAP* gene. Mutant *roc114-1* cells were transformed with *ROC114-TAP*, and bioluminescence rhythms of the chloroplast luciferase reporter (2) of spot cultures of the transformants were monitored in DD. Representative complemented rhythms are shown. A total of 1.2% (5/408) of transformants showed complemented circadian rhythms, indicating complementation of *roc114* by *ROC114-TAP*. (*D*) Representative result of Western blot analysis of the light response of ROC15-HA in a ROC114-TAP strain. Strains co-expressing ROC15-HA and ROC114-TAP were generated by genetic cross. Asynchronous cultures of the ROC15-HA/ROC114-TAP co-expressing strain were subjected to darkness for 9 h, and then exposed to a white light pulse (2 µmol m^{-2} s^{-1}, 0.5 min). Cells were harvested just before the light pulse and at 20 min after the onset of the light pulse.

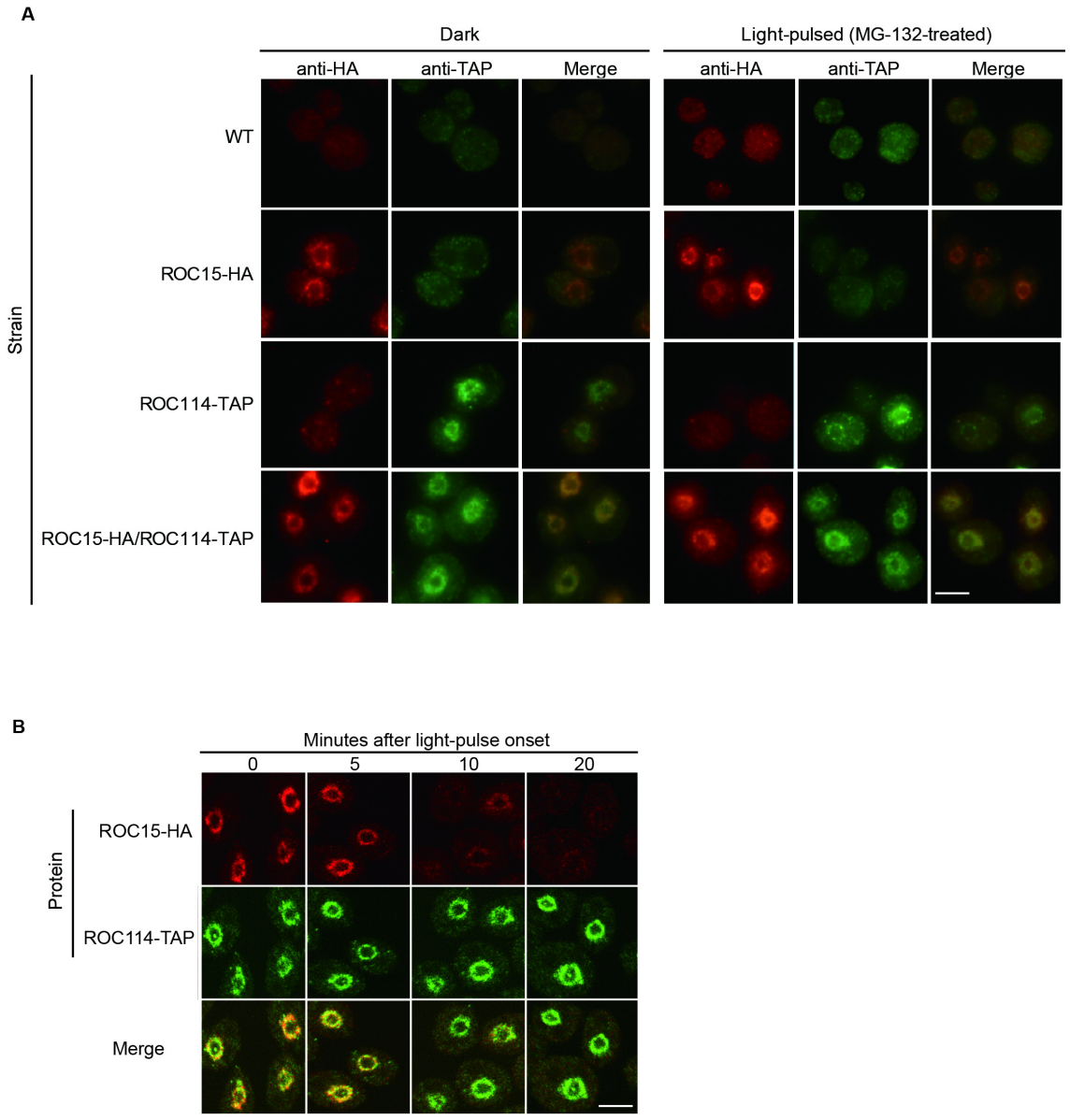

Fig. S13. Immunocytochemistry of ROC114-TAP. (*A*) Specificity of immunostaining. Asynchronous cultures of the ROC15-HA/ROC114-TAP co-expressing strain and control strains (WT, ROC15-HA, and ROC114-TAP) were subjected to darkness for 9 h, and then cells were harvested with or without exposure to a white light pulse (2 μmol m^{-2} s^{-1}, 1 min). The light-pulsed samples were pretreated with MG-132 (100 μM). Images shown were taken with a CCD camera. (*B*) Co-localization of ROC15-HA and ROC114-TAP. Asynchronous cultures of the ROC15-HA/ROC114-TAP co-expressing strain was subjected to darkness for 9 h, and then exposure to a white light pulse (2 μmol m^{-2} s^{-1}, 1 min). Cells were harvested at the time indicated. Confocal images are shown. Scale bars represent 5 μm (*A* and *B*).

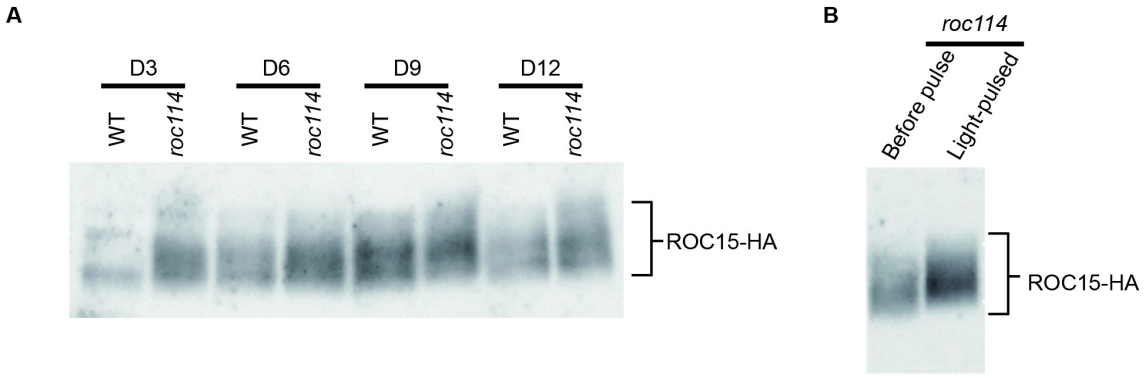

Fig. S14. Phosphorylation of ROC15 in the *roc114* mutant background. ROC15-HA strains having *roc114-1* genetic background were obtained by genetic cross. Cells were prepared as described in Fig. 1*B*, (A) Representative result of Western blot analysis of cells during the night phase. WT and *roc114* cells were harvested at the time point indicated. (B) Representative result of Western blot analysis of light-pulsed cells. A white light pulse (2 μmol m^{-2} s^{-1}, 0.5 min) was given at D9, and samples were harvested at 5 min after the light pulse.

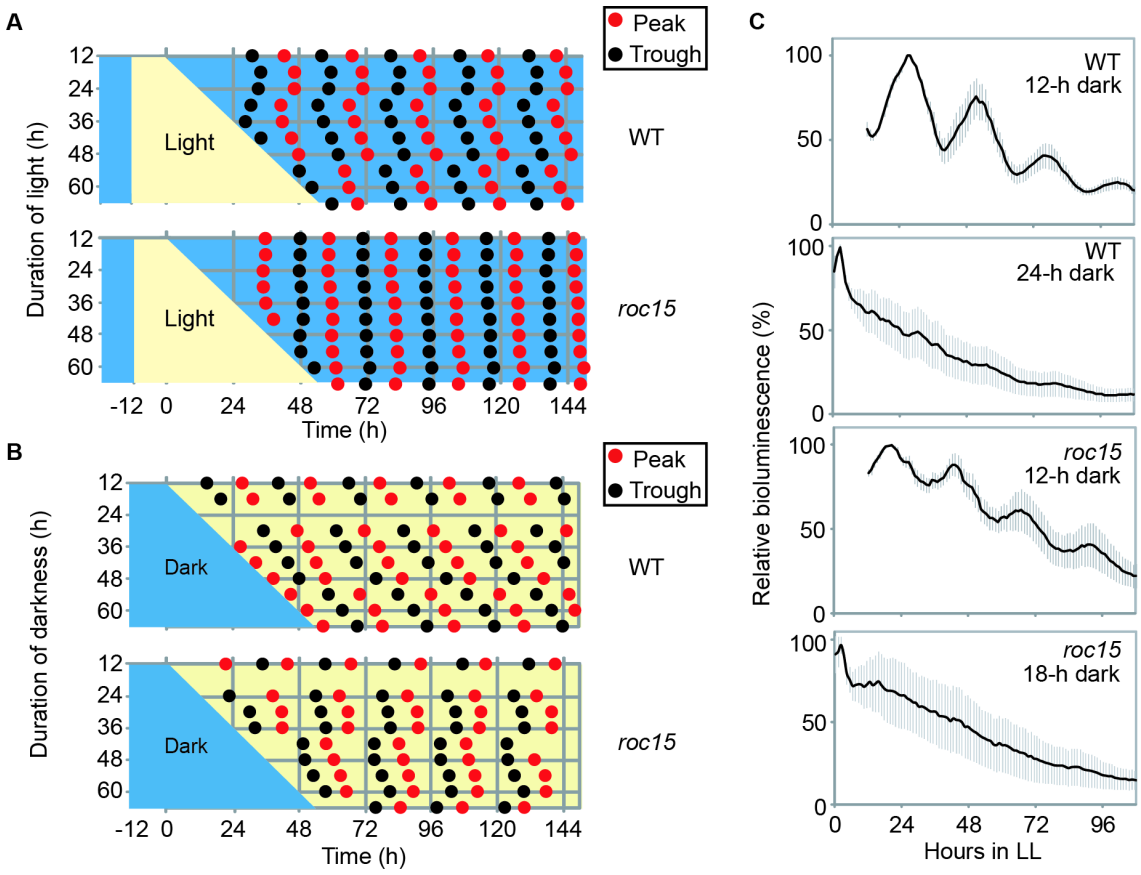

Fig. S15. The effects of extended light and dark exposures on the phase of subsequent circadian oscillations. (*A*) LL (25 µmol m^{-2} s^{-1})-grown asynchronous spot cultures were exposed to a 12-h dark treatment followed by different durations of light exposure (25 µmol m^{-2} s^{-1}). Bioluminescence rhythms of each sample were measured after transfer to DD. (*B*) LL (25 µmol m^{-2} s^{-1})-grown asynchronous spot cultures were exposed to different durations of darkness. Bioluminescence rhythms of each sample were measured after transfer to LL (2 µmol m^{-2} s^{-1}). The red and black plots indicate the average of peak and trough phases from 5-10 independent cultures. Peaks and troughs of WT and *roc15* cultures exposed to darkness for 24 and 18 h, respectively, were not plotted because the bioluminescence rhythms were hardly detected under these conditions. (*C*) Arrhythmic phenotypes of the chloroplast bioluminescence of WT and *roc15* cells after 24- or 18-h exposure to darkness. Representative arrhythmic traces are shown. The peak values of each bioluminescence trace were normalized to 100, and the mean ± SD of the normalized traces from 6–10 independent cultures are indicated. Similar results were obtained in 2 independent experiments (*A*-*C*).White light was used in all experiments in Fig. S15.

SI References

1. Matsuo T, et al. (2008) A systematic forward genetic analysis identified components of the *Chlamydomonas* circadian system. *Genes Dev* 22:918-30.
2. Matsuo T, Onai K, Okamoto K, Minagawa J, Ishiura M (2006) Real-time monitoring of chloroplast gene expression by a luciferase reporter: evidence for nuclear regulation of chloroplast circadian period. *Mol Cell Biol* 26:863-70.
3. Jeong Br BR, Wu-Scharf D, Zhang C, Cerutti H (2002) Suppressors of transcriptional transgenic silencing in *Chlamydomonas* are sensitive to DNA-damaging agents and reactivate transposable elements. *Proc Natl Acad Sci U S A* 99:1076-81.
4. Gorman DS, Levine RP (1965) Cytochrome *f* and plastocyanin: their sequence in the photosynthetic electron transport chain of *Chlamydomonas reinhardi*. *Proc Natl Acad Sci U S A* 54:1665-9.
5. Sueoka N (1960) Mitotic replication of deoxyribonucleic acid in *Chlamydomonas reinhardi*. *Proc Natl Acad Sci U S A* 46:83-91.
6. Matsuda Y, Saito T, Yamaguchi T, Kawase H (1985) Cell wall lytic enzyme released by mating gametes of *Chlamydomonas reinhardtii* is a metalloprotease and digests the sodium perchlorate-insoluble component of cell wall. *J Biol Chem* 260:6373-7.
7. Kubo T, Saito T, Fukuzawa H, Matsuda Y (2001) Two tandemly-located matrix metalloprotease genes with different expression patterns in the *Chlamydomonas* sexual cell cycle. *Curr Genet* 40:136-43.
8. Okamoto K, Onai K, Furusawa T, Ishiura M (2005) A portable integrated automatic apparatus for the real-time monitoring of bioluminescence in plants. *Plant Cell Environ.* 28:1305-1315.
9. Sizova I, Fuhrmann M, Hegemann P (2001) A *Streptomyces rimosus aphVIII* gene coding for a new type phosphotransferase provides stable antibiotic resistance to *Chlamydomonas reinhardtii*. *Gene* 277:221-9.
10. Okamoto K, Onai K, Ishiura M (2005) RAP, an integrated program for monitoring bioluminescence and analyzing circadian rhythms in real time. *Anal Biochem* 340:193-200.
11. Berthold P, Schmitt R, Mages W (2002) An engineered Streptomyces hygroscopicus *aph 7″* gene mediates dominant resistance against hygromycin B in *Chlamydomonas reinhardtii*. *Protist* 153:401-12.