The CONSTANS flowering complex controls the protective response of photosynthesis in the green alga *Chlamydomonas*

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Light is essential for photosynthesis, but the amounts of light that exceed an organism’s assimilation capacity can result in oxidative stress and even cell death. Plants and microalgae have developed a photoprotective response mechanism, qE, that dissipates excess light energy as thermal energy. In the green alga *Chlamydomonas reinhardtii*, qE is regulated by light-inducible photoprotective proteins, but the pathway from light perception to qE is not fully understood. Here, we show that the transcription factors CONSTANS and Nuclear transcription Factor Ys (NF-Ys) form a complex that governs light-dependent photoprotective responses in *C. reinhardtii*. The qE responses do not occur in CONSTANS or NF-Y mutants. The signal from light perception to the CONSTANS/NF-Ys complex is directly inhibited by the SPA1/COP1-dependent E3 ubiquitin ligase. This negative regulation mediated by the E3 ubiquitin ligase and the CONSTANS/NF-Ys complex is common to photoprotective response in algal photosynthesis and flowering in plants.
Photosynthetic organisms are often exposed to excess light. This can result in severe oxidative stress and even cell death. Plants and algae have developed a photoprotective response mechanism, qE, which is driven by low pH in the thylakoid lumen generated by the photosynthetic electron flow. This luminal low pH modifies the light-harvesting complex II pigment composition via the xanthophyll cycle and activates specific photoprotective proteins, such as PSBS and LIGHT-HARVESTING COMPLEX STRESS-RELATED PROTEINS (LHCSR). Mutants deficient in these effectors proteins are highly stressed under high light (HL) conditions. In a green alga Chlamydomonas reinhardtii, qE-type photoprotection is light-inducible via the expression of photoprotective proteins (LHCSR and PSBS). Two photoreceptors—PHOTOTROPIN (PHOT) and ultra-violet (UV) RESISTANCE LOCUS 8 (UVR8)—were identified as initiation factors for signal transduction of the photoprotective genes and protein expression in the green alga. Subsequently, two phot suppressor loci were identified to be involved in the induction of LHCSR; DE-ETIOLATED 1 (det1) and DAMAGED DNA-BINDING 1 (ddb1) and an E3 ubiquitin-conjugating ligase complex, CUL4–DDB1DET1, was proposed to mediate the PHOT signal to LHCSR genes expression. Despite these findings, we still do not know most of other important players in the signal transduction pathways, especially transcription factors that directly activate light-dependent gene expression. During a recent genetic screening using a bioluminescence reporter assay, however, we obtained several Deficient in LHCSR expression (DSR) mutants that showed reduced expression of LHCSR genes. Two of the mutants, DSR10 and DSR15, had a mutation in CONSTANS and two other mutants, DSR28 and CC4286, had a mutation in NF-YB.

CONSTANS (CO) is a circadian clock-regulated gene encoding a transcription factor required for flowering. Since Coupland et al. first reported that CO mediates photoperiodic flowering by directly regulating the transcription of FLOWERING LOCUS T (FT), extensive studies have revealed that CO and its ubiquitination under the control of photoreceptors are the central to regulation of photoperiodic flowering. NUCLEAR FACTOR Y (NF-Y) proteins (NF-YA, NF-YB, and NF-YC) bind to the promoter regions of their target genes and are widely conserved in eukaryotic organisms, including yeast, mammals, and plants. NF-Ys are the most thoroughly studied proteins that interact with CO. The biological functions and underlying molecular mechanisms of NF-Y proteins have been intensively investigated in both animals and plants. For example, Wenkel et al. reported that in flowering plants, HEME ACTIVATOR PROTEIN, which corresponds to NF-YA, strongly interacts with CO, forming a transcriptional complex that regulates the expression of its downstream gene, FT. The interaction of NF-YB2/3 and NF-YC3/4/9 with CO has physiological relevance in plants, as the absence of any of these proteins results in delayed flowering. A comparable sophisticated mechanism has not been identified in microalgae, as their population growth relies on cell division, not on flowering as in land plants.

Even though C. reinhardtii does not produce flowers, a copy of each of these transcriptional factors has been identified on its genome, including CONSTANS (CrCO), NF-YB, and NF-Y-C. Previously, heterologous expression of CrCO was shown to complement the function of CO in the flowering plant Arabidopsis thaliana. Phylogenetic analysis of CO and CO-like (COL) genes from green algae and land plants indicated that CrCO is a precursor of CO in green photosynthetic eukaryotes. The C. reinhardtii homologs of NF-YB and NF-YC were clustered with NF-YB1/8/10 and with NF-YC1/2/3/4/9, respectively, in phylogenetic analyses of the NF-Y protein family (Supplementary Fig. 1). Although few physiological and biochemical analyses of algal NF-Ys have been reported to date, these findings suggest that the CO/NF-YB/NF-YC transcriptional complex might have arisen before the divergence of land plants. In the current study, using the DSR mutants defective in CrCO or NF-YB and other newly constructed mutants in C. reinhardtii, we show that the transcription factors CrCO and NF-Ys form a complex that governs light-dependent photoprotective responses in C. reinhardtii. Further, we show that the CrCO/NF-Ys complex was directly inhibited by the SUPPRESSOR OF PHYA-105 I (SPA1) and CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1)–dependent E3 ubiquitin ligase. We propose that CrCO and its ubiquitination is the central to regulation of photoprotection in C. reinhardtii, much as like flowering in plants.

**Results**

CONSTANS and NF-YB are crucial for photoprotection. We identified mutations in CrCO in two DSR mutants, DSR10 (crco-1) and DSR15 (crco-2) (Supplementary Fig. 2a). A combination of linkage mapping and whole-genome sequencing revealed that two other mutants, DSR28 (nfyb-1) and CC4286 (nfyb-2), had mutations in NF-YB (Supplementary Fig. 2a, 2b). While DSR10 (crco-1) and DSR15 (crco-2) had reduced CrCO yet normal NF-YB transcript levels, DSR28 (nfyb-1) and CC4286 (nfyb-2) had normal CrCO yet reduced NF-YB transcript levels (Supplementary Fig. 2b). Similarly, RT-PCR analysis revealed that the transcripts of representative genes involved in photoprotection in C. reinhardtii (LHCSR and PSBS) were strongly reduced or almost undetectable in all of these mutants (Supplementary Fig. 3). Under 30 μmol photons m⁻² s⁻¹ (low light [LL]) and 100 μmol photons m⁻² s⁻¹ (medium light), these mutants had comparable growth rates and similar cell shapes to the wild-type strains. However, we obtained several Deficient in LHCSR expression (DSR) mutants that showed reduced expression of LHCSR genes. Two of the mutants, DSR10 and DSR15, had a mutation in CONSTANS and two other mutants, DSR28 and CC4286, had a mutation in NF-YB.

To test this hypothesis, we first measured photoprotective thermal dissipation of excess absorbed energy in the mutants. This process occurs via the quenching mechanism qE, which is the fastest reversible photoprotective response. It suppresses photos oxidative damage and contributes to optimizing photosynthetic efficiency under variable light conditions in nature. In C. reinhardtii, qE-type photoprotection is a light-inducible process that involves the expression of photoprotective proteins. When DSR15 (crco-2) and DSR28 (nfyb-1) strains were exposed to excess light (including weak UV), they exhibited pigment bleaching (Fig. 1a, b). This is an indicator of severe photoinhibition and is accompanied by a decrease in the maximum quantum efficiency of photosystem II (Fv/Fm) (Fig. 1c). These mutations were successfully rescued via expression of either CrCO (crco-2/GCrO) or NF-YB (nfyb-1/NFyB) fused with a YFP variant, Venus–3xFLAG (Fig. 1a–d). The photoinhibitory phenotype observed in crco-2 and nfyb-1 may have been due to insufficient expression of the key photoprotective proteins, including LHCSR1, LHCSR3, and PSBS, because the mutants’ qE activities were almost lost (Fig. 1d, e). These results indicate that both CrCO and NF-YB are essential for the functional activation of qE-dependent photoprotection in C. reinhardtii.

The kinetics of the photoprotective response of the rescued strains were then examined. The crco-2/GCrO strain showed a delayed response in both qE and photoprotective protein
CrCO and NF-YB are crucial for photoprotection. a The bleaching phenotypes of the reference strain (LHCSR1–Luc717) and the DSR mutants visualized in multiwell plates. Representative cell cultures treated with low light (LL; left wells) or high light (HL; right wells). Concentrations of the cultures were adjusted to $1.0 \times 10^7$ cells/mL. b Chlorophyll content per cell after LL (closed bar) or HL (open bar) treatment of the cells shown in a. c Maximum quantum yield of photosystem II ($F_\text{v}/F_\text{m}$) during HL treatment. d $q_E$ quenching capability during HL treatment. e Immunoblot analysis of 3xFLAG-fused proteins (CrCO–FLAG and NF-YB–FLAG in ccco-2/CrCO and nfyb-1/NFYB, respectively), LHCSR1, LHCSR3, and PSBS during HL treatment. ATPB protein levels are shown as the loading control. The experiments were performed three times with different biological samples ($n = 3$ biological replicates; mean ± S.E.M); a representative experiment is shown in e.
expression, whereas the nfyb-1/NFYB strain showed a normal photoprotective response (Fig. 1d, e). Considering that the expression kinetics of the key photoprotective proteins (LHCSRs and PSBS) are similar to CrCO protein accumulation (Fig. 1c), it is likely that the CrCO protein accumulation or localization in the cco/2/CrCO strain is altered. These results suggest that CrCO protein accumulation induced by light illumination was responsible for the photoprotective responses in C. reinhardtii.

CONSTANS/NF-Y complex controls photoprotective transcription. During the complementation analysis of cco-2 and nfyb-1, a Venus–3xFLAG tag was fused to the C termini of CrCO and NF-YB, which enabled intracellular live imaging of these proteins. Venus fluorescence was detected near the nucleus in the cco-2/CrCO and nfyb-1/NFYB strains. Immunocytochemistry analysis also showed that the DAPI and FLAG signals colocalized in the complemented strains (Supplementary Fig. 5), indicating that CrCO and NF-YB colocalized in the nucleus (Fig. 2a). Because complex formation among CO, NF-YA, and NF-YC has been reported to be essential for FT regulation in flowering plants20, we further tested the possibility of the involvement of NF-YC in the complex by generating the NF-YC (Cre12_g556400) mutants in C. reinhardtii. The CRISPR-Cas9-mediated mutation in NF-YC severely affected both the transcription and translation of the photoprotective factors, in a similar manner to the CrCO or NF-YB mutants. This resulted in cell death under HL (Supplementary Fig. 6). We subsequently tested the physical interactions between CrCO and NF-YB, CrCO and NF-YC, and NF-YB and NF-YC using yeast two-hybrid (Y2H) assays, suggesting that CrCO, NF-YB, and NF-YC interacted with each other (Fig. 2b and Supplementary Fig. 7). Similar tripartite interactions among the corresponding proteins have been reported in flowering plants15,20,21. These interactions were confirmed using immuno-coprecipitation assays of CrCO–Venus–3xFLAG and NF-YB–Venus–3xFLAG with FLAG monoclonal antibody followed by LC-MS/MS spectroscopy. This revealed the presence of NF-YB and NF-YC in the precipitates of CrCO–Venus–3xFLAG and NF-YB–Venus–3xFLAG, respectively (Supplementary Tables 1 and 2). Moreover, CrCO was detected in the precipitate of NF-YB–Venus–3xFLAG (Supplementary Fig. 8). Together, these results strongly indicate that the CO/NF-YB/NF-YC complex is conserved in C. reinhardtii.

Mutations in CrCO or NF-YB/NF-YC severely suppressed the transcription of the photoprotective genes in C. reinhardtii (Fig. 1 and Supplementary Fig. 6). Meanwhile, mutations in CO and/or NF-YA/NF-YB/NF-YC in flowering plants have been shown to reduce FT transcription10,15,20. All the photoprotective genes including LHCSR1, LHCSR3.1, and PSBS1 have at least one CO-responsive element (CORE), CCACA22, and NF-Y cis-element, CCAAT22, in the upstream regions of their start codons (Supplementary Fig. 9). It is therefore plausible that CrCO and NF-YB act as transcription factors that regulate the expression of the photoprotective genes in C. reinhardtii.

To investigate whether CrCO and NF-YB associate with the promoter regions of the photoprotective genes (LHCSR1, LHCSR3.1, and PSBS1) in C. reinhardtii, we performed chromatin immunoprecipitation (ChIP) assays using the cells before and after HL treatment. Both CrCO and NF-YB associated with the promoter regions of these genes after HL treatment (Fig. 2c), which was quantitated using ChIP-qPCR analysis (Supplementary Fig. 10). It has been suggested that the NF-YB/NF-YC complex in A. thaliana binds near CORE elements (CCCAAT for NF-YA22 and CCACA for CO22) to support the association of either NF-YA or CO with the promoter region22. Considering this binding feature of plant NF-YB/NF-YC, it is conceivable that NF-YB
weakly associates with the promoter region of the photoprotective genes under LL and that this association is reinforced by CrCO accumulation and the formation of a CrCO/NF-YB/NF-YC complex under HL. Together with the results of our physiological analysis (Fig. 1), this demonstrates that the CrCO/NF-YB/NF-YC transcriptional module is involved in photoprotective processes in *C. reinhardtii*.

**UVR8 inhibits E3 ubiquitin ligase to accumulate CONSTANS.** In land plants, CO protein accumulation is controlled by light-dependent post-transcriptional regulation [24]. This regulation is modulated by two CULLIN 4 (CUL4)-based E3 ubiquitin ligase components [25], SPA1 and COP1, which are involved in the ubiquitination-dependent degradation of CO [26,27]. The E3 ubiquitin ligase activity of this complex is deactivated by the formation of a protein–protein complex with photoactivated CRYPTOCHROME 2 (CRY2) [28]. The interaction between COP1 and the UV-photoreceptor UVR8 has also reported to be important for UV responses in both land plants [29] and *C. reinhardtii* [30]. Using a UVR8–Venus–3xFLAG expression strain, UV-inducible nuclear translocation of UVR8 was observed (Fig. 3a and Supplementary Figs. 5 and 11). Additionally, interactions among UVR8, SPA1, and COP1 were identified using a coimmunoprecipitation assay (Fig. 3b and Supplementary Tables 3 and 4). These results imply that upon UV perception, UVR8 deactivates E3 ubiquitin ligase through the formation of the UVR8/SPA1/COP1 protein complex as proposed in land plants [30].

As SPA1 and COP1 are involved in the ubiquitination-dependent proteasomal degradation of CO in land plants [26,27], we further investigated the possible interaction among SPA1, COP1, and CrCO. Both SPA1 and COP1 interacted with CrCO in Y2H assays (Fig. 3c). Additionally, UV-dependent accumulation of CrCO was observed in the crco-2/CrCO complemented strain, while it overexpressed CrCO mRNA even before UV illumination (Fig. 3d and Supplementary Fig. 12). It is therefore likely that CrCO is degraded in LL by proteasomes after ubiquitination by the SPA1/COP1-dependent E3 ubiquitin ligase complex. This degradation is then inhibited upon exposure to UV.

To further clarify whether the E3 ubiquitin ligase is involved in the accumulation of CrCO and photoprotective proteins, an *spa1* mutant was analyzed. This mutant has an insertional mutation in the SPA1 gene, and was obtained from the *Chlamydomonas* mutant library project (CLIP) (see Methods for detail). This mutation mimics deactivation of the SPA1/COP1-dependent E3 ubiquitin ligase. As expected, overexpression of the photoprotective proteins (LHCSRs and PSBS) were observed even under LL conditions in this mutant owing to the accumulation of CrCO (Fig. 3d). Furthermore, an inhibition of proteasome activity by MG132 treatment of the crco-2/CrCO strain, which resulted in the accumulation of CrCO protein even under LL conditions (Supplementary Fig. 13), also led to the accumulation of several ubiquitin polypeptides (*Cre09, g396400*), identified by LC-MS/MS analysis of the coimmunoprecipitated samples of CrCO–Venus–3xFLAG (Supplementary Table. 5). This indicates that CrCO is actively degraded by proteasomes that are guided by the ubiquitination of the protein. These data further support that SPA1/COP1 modules operate in distinct physiological functions, flowering time control in land plants and photoprotection in *C. reinhardtii*.

The CO/NF-YB/NF-YC complex plays a pivotal role in the photoperiodic control of flowering in land plants through transcriptional regulation of *FT* expression. Our findings...
therefore suggest that there may also be a link between photoperiodic signaling and the photoprotective response in *C. reinhardtii*. The transcript levels of *CrCO* indeed increased during subjective daytime but decreased in subjective night time (Supplementary Fig. 14). This is in part compatible with the previous report that *CrCO* transcription is under the control of the circadian clock \(^\text{16}\). Previous studies have also revealed that *LHCSR3*, formerly known as *LI818*, displays diurnal oscillations in RNA accumulation under light/dark cycles \(^\text{31,32}\). As *CrCO* is critical for survival under HL (Fig. 1), *CrCO* in fact connects the circadian clock with photoprotective responses in *C. reinhardtii* (Fig. 4).

### Discussion

Aihara et al. recently reported that another E3 ubiquitin ligase complex, CUL4–DDB1DET1, to be involved in the negative regulation of *LHCSR*s in *C. reinhardtii* \(^\text{8}\). Since a similar complex has been proposed to enhance the activity of the SPA1/COP1-dependent E3 ubiquitin ligase in land plants \(^\text{25}\), the algal CUL4–DDB1DET1 may also enhance the ubiquitination at the SPA1/COP1-dependent E3 ubiquitin ligase, thereby transducing the blue light signal to *LHCSR*s by way of *CrCO*. Taken together with the synchronized accumulation kinetics of *CrCO* and the photoprotective proteins in the *crco-2/CrCO* strain (Fig. 1e), we propose that the post-translational regulation of *CrCO* mediated...
by the E3 ubiquitin ligase, is fundamental for the photoprotective response in *C. reinhardtii* (Fig. 4). The initial light input signals differ between flowering plants (in which blue light is perceived by CRY2) and *C. reinhardtii* (in which UV is perceived by UVR8). Despite this, the kernel of the downstream regulatory components (SPA1/COPI-dependent E3 ubiquitin ligase to CO/NF-Ys transcriptional complex) is shared (Fig. 4). Previously, the regulatory kernel has been proposed as part of a unique mechanism developed for photoperiodic flowering. The results presented in this study have shown that the kernel’s counterpart in *C. reinhardtii* functions in photoprotection. This raises the possibility that the root of this method of regulation could be extended back to the early evolutionary history of green photosynthetic organisms. At the same time, we may need to reinterpret the significance of flowering in plants and photoprotection in green algae within the same context, because of a shared fundamental regulatory mechanism. Whether we can see the same type of regulation in other algae would be a matter of future study as our current knowledge about their photoprotective behavior as well as the related genetic information is still very limited.

### Methods

**Statistics.** Statistical methods were not used to determine the sample size. The experiments were not randomized, and the investigators were not blinded to allocation during experimental procedures and data assessment.

**Strains and growth conditions.** *Chlamydomonas reinhardtii* strains 137c, CC4286, and *C. tepidum* (CC3325 as the wild type and LM0002006430 as the sp1 mutant) were obtained from the Chlamydomonas Resource Center (http://www.chlamycol.org). All strains were grown in tris-acetate-phosphate (TAP) medium and illuminated at 50 μmol photons m⁻² s⁻¹ at 25 °C with rotary shaking at 150 rpm. Cells at mid-log phase (4 × 10⁵ cells/mL) were harvested and resuspended in fresh medium at 1 x 10⁶ cells/mL. The cell density was described in the text and figure legends. The LHCSR1–Luc717 strain, harboring a LHCSR1–luciferase fused reporter construct, was used as a control strain for the dsr mutants. The 137c strain was used as a control/recipient strain for NF-YC mutants. All the DSRI mutants were previously isolated through a forward genetic screen using the LHCSR1–Luc717 strain.

**Growth evaluation of the strains.** The growth of WT and mutant strains used in this study was evaluated under the conditions previously described. Brieﬂy, cells were grown under 30 or 100 μmol photons m⁻² s⁻¹ of continuous white fluorescent light in HS media, and cells were counted every 24 h. To assess cell shapes, cells at the steady-state growth phase (day 6) were imaged at room temperature using a TCS SP8 confocal laser scanning microscope (Leica Microsystems, Germany) equipped with a HCX PL APO CS2 63x/1.0 objective lens. A 440-nm diode laser with 2% output was selected to obtain bright-field images. The images were produced on a PIMP-based detector. All images were acquired with a 600-Hz laser scan speed and analyzed by LASX software (Leica Microsystems).

**Identification of the insertion site of the aph7 cassette.** The genomic regions flanking the inserted DNA tag were cloned by RESDA-PCR. The tag DNA-mixture-specific and degenerate primers are listed in Supplementary Table 1. The PCR mixture for a first amplification contained tag-specific primer (aph7tag-F1 or aph7tag-F2 for downstream of the tag DNA, aph7tag-R1 or aph7tag-R2, respectively, and genomic DNA as template) that were designed to amplify a 33-bp deletion (Supplementary Fig. 2). The primers were used with a β-lactamase gene as an object beacon. A 440-nm diode laser with 2% output was selected to obtain bright-field images. The images were produced on a PIMP-based detector. All images were acquired with a 600-Hz laser scan speed and analyzed by LASX software (Leica Microsystems).

**Linkage mapping of the dsr286 and dsr28 mutations.** Having determined that the CC4286 strain harbored a mutation that caused a defect in UV-inducible LHCSR1 expression, *C. reinhardtii* was named the maize mutant dsr286. The position in the 137c background, CC4286 was crossed with LHCSR1–Luc443, another clone of *LHCSR1–luciferase transfectants of 137c (mt-) and Luc443-1A-12-2b (mt+), was crossed with the polytrophic strain S1D2 (mt−, CC22950, and tetrad progeny were dissected. Recombination frequencies between the *dsr286* gene markers were determined by detecting polymorphic PCR products in 44 progeny that were deficient in UV-inducible LHCSR1 expression (dsr286 pheno-type). *dsr286* was mapped to a region before position 1515000 on chromosome 2 of the Joint Genome Institute (JGI) version 5.5. To examine the linkage between the *dsr286* and *dsr28* mutation, *dsr286*-*dsr28* combination was created with an insertion of a backcross of DSR28 and LHCSR1–Luc717 (mt−). All the resulting 91 progeny were tested for LHCSR1 expression and showed the mutant pheno-type, suggesting that mutation in *DSR28* (dsr28) is strongly linked to *dsr286*.

**Whole-genome sequencing of dsr286 and dsr28.** Two *dsr286* mutants, the F3 progeny of a backcross of CC4286 (#443-1A-12-5a (dsr286 mt+) and #443-1A-12-5d (dsr286 mt−)), and four clones from one tetrad set of the second backcross of DSR28 (#DSR28-7c-a (dsr28 mt−), –b (wild-type mt+), –c (wild-type mt+), and –d (mt+)) were subjected to whole-genome sequencing analysis. The genomic DNA was prepared using a DNeasy Plant Mini Kit (Qiagen, Germany). Libraries were constructed using an Illumina TruSeq DNA PCR-Free Library Prep Kit (Illumina, San Diego, CA, USA) from 2 μg of each DNA sample, according to the manufacturer’s protocol. The library sequence data was submitted to the DDBJ Sequence Read Archive (SRA) (http://www.ddbj.nig.ac.jp/dra). All sequences were trimmed with Trimmomatic and aligned onto the JGI version 5.5 *Chlamydomonas* assembly using Bowtie2 (bowtie.bio.sourceforge.net/­bowtie2/index.shtml). The resulting alignment data were converted to Binary Sequence Alignment/Map and sorted by SAMTools (sambtools.sourceforge.net/). The variants were identified using SAMTools and freebayes (https://github.com/ekg/freebayes/).

**Whole-genome sequencing of dsr286 and dsr28.** Two *dsr286* mutants, the F3 progeny of a backcross of CC4286 (#443-1A-12-5a (dsr286 mt+), #443-1A-12-5d (dsr286 mt−), and #DSR28-7c-c (dsr28 mt−), –b (wild-type mt+), and #DSR28-7c-c (wild-type mt−)) were selected for the whole-genome sequencing analysis. The genomic DNA was prepared from strains #443-1A-12-5a and #443-1A-12-5d as a template. Twenty variants were identified that were present in #443-1A-12-5a and #443-1A-12-5d but not in DSR28-7c-c (wild-type mt−) or DSR28-7c-c (wild-type mt+). These variants were further confirmed by PCR analysis and were mapped to a region before position 1515000 on chromosome 2 of the Joint Genome Institute (JGI) version 5.5. To examine the linkage between the *dsr286* and *dsr28* mutation, *dsr286*-*dsr28* combination was created with an insertion of a backcross of DSR28 and LHCSR1–Luc717 (mt−). All the resulting 91 progeny were tested for LHCSR1 expression and showed the mutant pheno-type, suggesting that mutation in *DSR28* (dsr28) is strongly linked to *dsr286*.

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**NYFC gene disruption using the CRISPR-Cas9 system.** CRISPR-Cas9 targeting of NYFC exon2 was performed to generate nyfc mutants as described previously, using a guide RNA sequence (5’TGAAATACGTGTTGGTACGTT-3’) and a double-stranded homology-directed repair donor (ds-HDR). The sequences of (HDF-R) and antisense (HDR-R) 50-bp oligonucleotides were arranged with three PTO bonds at the 3' and 5' end bases. Equimolar concentrations of sense and antisense strand 50-bp oligonucleotides were mixe
concentrated cells were heat-shocked at 40 °C for 30 min and mixed using a thermoshaker (350 rpm). Forty microliters of heat-shocked cell suspension was mixed with 3 μl of 10 mM RNase A, 1.5 μl of 10 μM dE-DHFR, and 0.3 μg of selection marker plasmid pHyg3 and then electroporated using the NEPA21 Super Electroporator (Nepa Gene Co., Ltd., Japan). The transformed cells were then selected under dim light at 25 °C on TAP plates containing 10 μg/ml of hygromycin. The hygromycin-resistant clones were screened for insertion of the “FLAG” sequence by colony PCR using NPYC-check-F and FLAG-R primers.

Complementation of DSR mutants. For the complementation of cre2-2 (DSR15), nfyb-1 (DSR28), and uvrb (DSR1) mutants, a full-length version of their genes was fused in-frame to Venus-3xFLAG under the control of the hisP70/2RBC2 tandem promoter45. The generated complementation vectors with the adaA gene (speci- nomycin resistance marker) were introduced into the DSR15, DSR28, or DSR1 strains by electroporation using a NEPA21-Nepa Gene Co Ltd. All strains in the exponential phase of growth (2 × 10^6 cells/ml) were concentrated cells were heat-shocked at 40 °C for 30 min and mixed using a Bioruptor (BIO-CRAFT, Japan) at a constant voltage 40 V for 3 h. The blotted membranes (BIO-CRAFT, Japan) at a constant voltage 40 V for 3 h. The blotted membranes were treated with a blocking reagent EzChemi Block (ATTO, Japan) for 1 h. The primary and secondary antibody treatments were done with shaking at room temperature for 15 min, and the eluted polypeptides were separated on a 12.5% polyacrylamide gel. The proteins sizes were scanned and cropped using the EzWestLumi Plus (ATTO), and images of the blots were graphed, as indicated in the figure captions (Fig. 1 and Supplementary Fig. 6).

Genetic crossing of the mutants. To generate double mutants harboring the spa1 mutation and CrCo-Venus-3xFLAG construct, the ClIP spa1 mutant LMI (Y4042-006-0040) was crossed with the cre2-2/COO, spa1-2, and spa1 cre2-2/CCO genotype strains. To generate triple mutants harboring the cre2-2 mutation, nfyb-1 mutation, and NFBY-Venus-3xFLAG construct, the cre2-2 mutant was crossed with the nfyb-1/NFBY strain. The resulting progeny were dissected and cre2-2 nfyb-1/NFBY genotype strains were isolated.

Standard mRNA quantification. Total RNA from light-treated cells was extracted using the Maxwell RSC instrument (Promega) equipped with the Maxwell RSC simplyRNA Tissue Kit (Promega). The isolated RNA was quantified using the QuantiFlour RNA System (Promega) prior to reverse transcription. Reverse transcription and semiquantitative PCR were performed using a ReverTra Ace® qPCR RT Kit (Toyobo) and KOD FX Neo DNA Polymerase (TOYOBO) in the SimpliAmp Thermal Cycler (Thermo Fisher Scientific). For reverse transcription, a 300 ng of isolated RNA sample was used in a 10-μl total reaction volume. Semiquantitative RT-PCR was performed using 3 ng of cDNA thus obtained. For semiquantitative RT-PCR, the G protein β subunit-like polypeptide (CBlP) gene was chosen as the housekeeping gene during light treatment. The primers used for semiquantitative RT-PCR are listed in Supplementary Table 7.

Immunoblot analysis. Protein samples from whole cell extracts (corresponding to ~2 × 10^6 cells, unless stated otherwise) were loaded onto 11% acrylamide with 7 M urea SDS-PAGE (gel size: 15 cm width × 10 cm height × 1 mm thickness), and the electrophoresis was performed at a constant current 8 mA for 15 h. The separated polypeptides were blotted onto nitrocellulose membranes with a tank blot system (Bio-Dot, Japan) at a constant voltage 40 V for 3 h. The blotted membranes were treated with a blocking reagent EzChemi Block (ATT0, Japan) for 1 h. The primary and secondary antibody treatments were done with shaking at room temperature for 1 h. Antiserum against ATPB was obtained from Agrisera (AS05 085, rabbit polyclonal, at 1:10,000 dilution); antiserum against LHSRCR and LHSRCS (rabbit polyclonal, at 1:10,000 dilution) were raised and affinity purified against the peptide LGLKQIDPEELF42 antiserum against PSBS (rabbit polyclonal, at 1:5000 dilution) was obtained from Prof. Peter Jahnis (Heinrich-Heine University, Germany); antiserum against CrCo (rabbit polyclonal, at 1:1000 dilution) was raised and affinity purified against the peptide AAWFVDVEKMG (Eurosinfo Genomics, Japan); and antiserum against FLAG fusion proteins was obtained from Sigma-Aldrich (F8040, mouse monoclonal, at 1:1000 dilution). An anti-rabbit horseradish peroxidase-conjugated antigen (70747, Cell Signaling Technology, Danvers, MA, USA) or an anti-mouse horseradish peroxidase-conjugated antigen (3330, MBL, Japan) was used as a secondary antibody (at 1:10,000 dilution). The blots were developed using the EzWestLumi Instant Imaging Kit (ATTO). The immunoprecipitates were then eluted using a CCD camera ImagerDocTouch System (Bio-Rad Laboratories, Hercules, CA, USA). The upper band of LHSRCR represents its phosphorylated form (LHSRCR3-P).
Immunocytochemistry of Venus-3xFLAG fusion proteins. Cells were harvested by centrifugation at 2000 x g at 4 °C for 2 min, and fixed by suspension in PBS containing 1% paraformaldehyde at 4 °C. The fixed cells were seeded on poly-L-lysine-coated coverslips (Asahi Techno Glass Corp., Japan) and were treated with methanol twice each for 10 min at −20 °C to permeabilize the membrane and remove chlorophyll. After rehydration with PBS, the coverslips were treated with a blocking buffer (5% bovine serum albumin, 1% cold water fish skin gelatin, and 10% goat serum in PBS), a primary antibody (mouse monoclonal anti-FLAG (M2) antibody; Sigma-Aldrich) at 4 °C overnight, and a secondary antibody [AlexaFluor546-conjugated F(ab’)2 fragment of goat anti-mouse IgGc. Thermo Fisher Scientific®] at room temperature for 2 h. Coverslips were then mounted onto the slides with ProLong Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific®). Fluorescence was observed with a confocal laser scanning fluorescence microscope (EV10i-DOC, Olympus, Japan).

Proteasome inhibitor assay. The proteasome inhibitor MG132 (Wako Chemical) dissolved in dimethylsulfoxide (DMSO) was added to the culture before starting the light treatment. The final concentration of DMSO was adjusted to 0.5% with a final concentration of MG132 of 0, 10, 25, 50, 100, and 200 μM, as indicated in Supplementary Fig. 13. The cells were cultured under LL for 2 h after MG132 was added.

Circcadian mRNA rhythm assay of CrCO. Asynchronous cultures in HS media (2 x 10^6 cells/mL) were kept in darkness for 12 h at 17 °C to synchronize the circadian clock and then exposed to continuous light (10 μmol photons m^{-2} s^{-1}). Cells were collected at intervals from 24 to 72 h. mRNA quantification by RT-qPCR was performed as described previously. Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific), ReverTra Ace® qPCR RT Master Mix (TOYOBO) was used for the reverse transcription procedures. qPCR was then performed using KOD FX Neo DNA polymerase (TOYOBO) supplemented with SYBR® Green I Nucleic Acid Stain (Thermo Scientific®) and ROX® (Thermo Fisher Scientific®) to final concentrations of 0.5 x (200,000-fold dilution) and 0.5 μM, respectively. Samples were quantified using StepOnePlus™ (Thermo Fisher Scientific®) by the relative standard curve method. Quantification standards for each target cDNA were obtained using band-purified PCR products as templates. The constitutively expressed RCK1 gene was used as an endogenous control for normalization. The primers used for PCR are listed in Supplementary Table 7.

Phylogenetic analysis of NF-Y proteins. The whole amino acid sequences of the NF-Y proteins from C. reinhardtii and A. thaliana were aligned using MAFFT v7.407 (https://mafft.cbrc.jp/alignment/software/). The phylogenetic tree was generated using RAxML v8.2.12 (https://cme.h-its.org/exelixis/web/software/raxml/index.html) with a 1000 bootstrap test. The generated phylogenetic tree was constructed and visualized with MEGA7 (https://www.megasoftware.net/).

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**Author contributions**

R.T. and J.M. conceived the research. R.T. designed the experiments. R.T., K.F-K. and T.Y. screened and identified the mutation sites of the mutants. R.T. performed the transcriptional, ChIP-PCR, chlorophyll fluorescence, pigment bleaching, live-cell imaging, biochemical, and phytogenetical analyses. K.F-K. performed the algal linkage mapping, whole-genome sequencing, yeast two-hybrid analyses, and mutant generation. T.M. performed the immunocytochemistry and analyzed the circadian clock response of the green alga. R.T. wrote the initial draft of the paper. J.M. supervised the entire work and revised the paper. All authors contributed to the writing and revision of the paper and approved the final version of the paper.

**Additional information**

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