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Regulation of photoprotection gene expression in *Chlamydomonas* by a putative E3 ubiquitin ligase complex and a homolog of CONSTANS

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Photostressive organisms use nonphotochemical quenching (NPQ) mechanisms to dissipate excess absorbed light energy and protect themselves from photooxidation. In the model green alga *Chlamydomonas reinhardtii*, the capacity for rapidly reversible NPQ (qE) is induced by high light, blue light, and UV light via increased expression of LHCSR and PSBS genes that are necessary for qE. Here, we used a forward genetics approach to identify SPA1 and CUL4, components of a putative green algal E3 ubiquitin ligase complex, as critical factors in a signaling pathway that controls light-regulated expression of the LHCSR and PSBS genes in *C. reinhardtii*. The *spa1* and *cul4* mutants accumulate increased levels of LHCSR1 and PSBS proteins in high light, and unlike the wild type, they express LHCSR1 and exhibit qE capacity even when grown in low light. The *spa1-1* mutation resulted in constitutively high expression of LHCSR and PSBS RNAs in both low light and high light. The qE and gene expression phenotypes of *spa1-1* are blocked by mutation of *CrCO*, a B-box Zn-finger transcription factor that is a homolog of CONSTANS, which controls flowering time in plants. CONSTANS-like cis-regulatory sequences were identified proximal to the qE genes, consistent with *CrCO* acting as a direct activator of qE gene expression. We conclude that SPA1 and CUL4 are components of a conserved E3 ubiquitin ligase that acts upstream of CrCO, whose regulatory function is wired differently in *C. reinhardtii* to control qE capacity via cis-regulatory CrCO-binding sites at key photoprotection genes.

light harvesting | light signaling | nonphotochemical quenching | photomorphogenesis | photosynthesis

Light is necessary for photosynthesis, but the supply of light in natural environments is not constant. Photostressive organisms have evolved functional flexibility in their light-harvesting systems, enabling efficient absorption and utilization of limiting light and photoprotection in excess light. In saturating light, photostressive light harvesting is regulated by nonphotochemical quenching (NPQ) mechanisms that are responsible for dissipating excess absorbed light energy as heat (1, 2).

NPQ involves the deexcitation of singlet excited chlorophyll (1Chl) in photosystem II (PSII), and the term NPQ reflects the way in which these processes are routinely assayed through measurements of chlorophyll fluorescence quenching (3). The major component of NPQ under most short-term light stress conditions is called qE (1, 2, 4). It is turned on and off rapidly (seconds to minutes) and depends on the formation of a large ΔpH across the thylakoid membrane in excess light (5). In the reference green alga *Chlamydomonas reinhardtii*, a light-harvesting complex (LHC) protein called LHCSR functions as both a sensor of lumen pH and a site of qE to which pigments are bound (6–9). *C. reinhardtii* has 3 genes encoding LHCSR: LHCSR1, LHCSR3.1, and LHCSR3.2 (6). In plants, another member of the LHC protein superfamily, PSBS, is a major sensor of lumen pH (10) that turns qE on and off by somehow facilitating a rearrangement of LHC proteins that surround PSII (11–15). PSBS also appears to contribute to qE in *C. reinhardtii* (16–18), which has 2 closely linked PSBS genes, PSBS1 and PSBS2 (19).

LHCSR expression and qE capacity in *C. reinhardtii* are inducible by exposure to high light (6), UV light (18), and blue light (20), whereas cells grown in low light have a very limited qE capacity (6). Induction of LHCSR expression and qE involves photoreceptors such as UVR8 (18, 21) and phototropin (PHOT) (20), however, the signaling components that act downstream of the photoreceptors to regulate LHCSR expression have not been elucidated (22). Here, we describe the results of a genetic screen in which we isolated mutants that overexpress LHCSR1 and PSBS. These mutants have an increased capacity for qE even when grown in low light, and they show that a conserved E3 ubiquitin ligase complex (23–25) is involved in the regulation of LHCSR and PSBS gene expression via an ortholog of the plant transcription factor CONSTANS (26).

Results

Identification and Genetic Analysis of Suppressors of *npq4*. We performed a suppressor screen designed to isolate mutants that

Significance

Photoprotection is an indispensable component of abiotic stress tolerance in all photosynthetic organisms. In contrast to land plants, the transcript abundance of photoprotection genes is strongly controlled by light in the model green alga *Chlamydomonas reinhardtii*. Here we show that the repression of photoprotection genes in low light depends on 2 putative components of a conserved E3 ubiquitin ligase complex. We also show that this regulation of gene expression depends on a transcription factor that is conserved among plants and algae and that acts downstream of the putative E3 ubiquitin ligase complex. Our work provides mechanistic insight into how a deeply conserved light signaling pathway is wired differently in *C. reinhardtii*, giving rise to light control of photoprotection gene expression.

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Data deposition: Genomic sequencing data have been deposited in the NCBI BioProject database with the BioProject ID PRJNA553556. Other data are available from the corresponding author upon request.

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overaccumulate LHCSR1 and/or PSBS by UV mutagenizing the C. reinhardtii npq4 mutant and screening for colonies with increased NPO using video imaging of chlorophyll fluorescence (27). The npq4 mutant exhibits low qE capacity, because it lacks functional LHCSR3.1 and LHCSR3.2 genes (6), but it retains intact copies of LHCSR1, PSBS1, and PSBS2. Therefore, we hypothesized that overexpression of one or more of these genes would be a simple mechanism for increasing qE in npq4. Among ∼10,000 mutagenized colonies, 30 mutants showed reproducibly elevated NPO values relative to the npq4 parent strain. Fig. 1 shows the kinetics of NPO induction and relaxation for 3 npq4 suppressors (spa1-1, spa1-2, and cul4-1) that we selected for further analysis. These suppressors showed NPO levels that were intermediate between the npq4 parent and the wild-type (WT) strain after growth in high light (HL) (Fig. 1B), and they also displayed higher NPO relative to npq4 and the WT strain after growth in the noninducing condition of low light (LL) (Fig. 1A). The NPO observed in these 3 suppressors was rapidly reversible in the dark (Fig. 1), indicating that the qE component of NPO was increased.

To determine if the npq4 suppressors overaccumulate LHCSR1 and/or PSBS, we examined levels of these proteins by immunoblot analysis of LL- and HL-grown cells. In WT, LHCSR protein was only detected in HL-grown cells (SI Appendix, Fig. S1). In contrast, the npq4 suppressors accumulated high levels of LHCSR1 in both inducing (HL) and noninducing conditions (LL), and the LHCSR1 levels in HL were elevated relative to those observed in npq4. We were also able to detect PSBS in the 3 npq4 suppressors when grown in HL (SI Appendix, Fig. S1). This was an unexpected result given that detection of PSBS in C. reinhardtii has previously been observed only transiently in stress conditions such as during the first hours of exposure to very high light (16), damaging levels of UV-B radiation (18), or HL in combination with low CO2 (17).

Backcrossing the 3 npq4 suppressors to npq4 revealed a 2:2 segregation of the NPO phenotype (SI Appendix, Fig. S2), showing that the high NPO phenotype is the result of a single nuclear mutation in each of these 3 lines from the npq4 mutation by crossing to WT. Analysis of progeny of tetratype tetrads showed that the high NPO phenotype of the suppressor mutations in LL- and HL-grown cells was also evident in a WT background (SI Appendix, Fig. S3).

Molecular Analysis of spa1 and cul Mutations. The causative suppressor mutations were identified by a combination of whole-genome sequencing and bulked segregant analysis of backcross progeny (Fig. 2A). Briefly, the NPO values of backcross progeny were quantified (SI Appendix, Fig. S2), and pools of low and high NPO progeny were constructed and sequenced, along with the parents. In both spa1-1 and spa1-2, the putative causative polymorphisms were mapped to independent missense mutations at conserved residues in Cre13.g02700, an ortholog of Arabidopsis thaliana SPA1 (Fig. 2B and SI Appendix, Fig. S4 and Table S1). The putative causative polymorphism in cul4-1 was identified as a missense mutation at a conserved residue in Cre12.g516500, an ortholog of A. thaliana CUL4 (Fig. 2C and SI Appendix, Fig. S4 and Table S2). We confirmed that expression of a wild-type version of SPA1 rescued the NPO phenotype of npq4 spa1-1 (SI Appendix, Fig. S5). Of the 3 suppressors, spa1-1 was selected for complementation and further analysis, because it exhibited the strongest induction of NPO in LL (SI Appendix, Fig. S3).

In A. thaliana, both SPA1 and CUL4 are subunits of the COP1-SPA E3 ubiquitin ligase complex (28) that is involved in photomorphogenesis (23–25). SPA1-SPA4 and CUL4 are paralogous WD40 repeat domain-containing proteins, and 2 copies of SPA and 2 copies of CUL1 form a heterotrimer (29), which acts as the substrate acceptor domain of the E3 ubiquitin ligase complex (28). Unlike COP1, SPA proteins contain a Ser/Thr kinase-like domain (Fig. 2B), which has been shown to have a regulatory role in modulating the activity of the complex (30, 31). In C. reinhardtii, which has a single SPA gene, the spa1-1 mutation maps to the Ser/Thr kinase-like domain (SI Appendix, Fig. S4A), whereas spa1-2 maps to the WD40 repeat domain (SI Appendix, Fig. S4B). Notably, missense mutations in the Ser/Thr kinase-like domain of SPA1 in A. thaliana induced a loss-of-function phenotype (30). CUL4 acts as a scaffold protein within A. thaliana CUL4-DDB1 E3 ubiquitin ligase complexes, connecting the COP1-SPA substrate acceptor domain to the E3 ubiquitin ligase (28). CUL4 contains both Cullin and winged helix protein interaction domains (Fig. 2C), and the cul4-1 mutation in C. reinhardtii maps to the Cullin domain (SI Appendix, Fig. S4C).

The C. reinhardtii Homolog of CONSTANS Acts Downstream of SPA1. Using de novo motif identification software (32), we identified a promoter sequence, shared within ∼350 bp of the translation start site of all 5 of the qE genes (LHCSR1, LHCSR3.1, LHCSR3.2, PSBS1, and PSBS2) in C. reinhardtii (Fig. 3). This motif with a CCACA core strongly resembles the binding site for the plant transcription factor CONSTANS (CO) (33), a well-known substrate protein that COP1-SPA targets to the E3 ubiquitin ligase complex in A. thaliana (34–36). C. reinhardtii has a homolog of CO, called CrCO, with a conserved function in circadian gene regulation and a conserved DNA-binding specificity as demonstrated by complementation of the A. thaliana co mutant by CrCO (26). We previously identified a mutant (CAL028_01_08) affected in CrCO

![Fig. 1](image-url)

Three suppressors of npq4 have elevated NPO capacity when grown in LL (60 μmol photons m−2 s−1) or HL (350 μmol photons m−2 s−1). NPO was induced with actinic light of 600 μmol photons m−2 s−1 (white bar at Top), followed by recovery in the dark with 0.6 μmol photons m−2 s−1 far-red light (black bar at Top). (A) LL-grown cells. (B) Cells grown in HL for 3 d. Values represent means ± SD (n = 3).
support the existence of a SPA1-independent, posttranscriptional regulatory mechanism that controls protein levels of LHCSR3 and PSBS, but not LHCSR1, in a light-dependent manner.

Discussion

In this work, we demonstrate that SPA1 and CUL4, subunits of the deeply conserved COP1-SPA1 E3 ubiquitin ligase complex, are necessary for the repression of qE capacity in LL-grown C. reinhardtii (Fig. 5). Furthermore, we show that CrCO, an ortholog of the A. thaliana CO transcription factor, is necessary for the expression of LHCSR and PSBS genes and that it is downstream of SPA1-mediated control of qE gene expression and qE capacity. By analogy with A. thaliana, our data strongly indicate that the COP1-SPA1 complex serves as a substrate receptor for a CUL4-DDB1 E3 ligase (28) to control degradation of CrCO in response to light signals in C. reinhardtii (Fig. 5). However, in contrast to A. thaliana, the target genes of CrCO are wired differently with CO-like binding sites in the promoter sequences for qE genes, thus ultimately placing photoprotection under the control of COP1-SPA1 E3 ubiquitin ligase-mediated signaling in C. reinhardtii. Based on analysis of promoters (Fig. 3) and transcript levels (Fig. 4), we suggest that CrCO regulates qE gene transcription through direct promoter binding (Fig. 5). We also find evidence that there is at least 1 other pathway that operates independently of SPA1 and CUL4 to control the HL induction of gene expression for these qE genes (Figs. 4 and 5). Finally, our work also highlights that there are mechanisms beyond transcriptional control that are necessary to explain the complexity of protein abundance for LHCSR3 and PSBS.

It was recently reported that another E3 ubiquitin ligase complex is involved in regulating LHCSR1 and LHCSR3 expression in C. reinhardtii (38). In this work, mutations in DET1 and DDB1, which encode 2 subunits of a putative CUL4-DDB1 E3 ligase complex, were shown to lead to accumulation of LHCSR1, LHCSR3, and PSBS transcripts in the dark, similar to what we observed in LL. In A. thaliana, DET1 E3 ligase complexes are distinct in composition from the COP1-SPA1 complex, although CUL4 and DDB1 are subunits of both types of complexes (39). We propose 2 plausible models to synthesize our results with those found for the DET1 complex. In 1 model, the DET1 and the COP1-SPA1 E3 ubiquitin ligase complexes are both necessary to

(Cr06g278159) in our collection of DNA insertional mutants (37). Analysis of PCR products across the gene showed that the mutant has a deletion affecting the CrCO gene, whereas the flanking genes were intact (SI Appendix, Fig. S6A and B). The crco mutant exhibited a severe defect in NPQ, specifically qE (Fig. 4), that cosegregated 2:2 with the paromomycin resistance cassette used for insertional mutagenesis (SI Appendix, Fig. S6C), and the mutant was sensitive to HL (SI Appendix, Fig. S7). Additionally, the low NPQ phenotype of the crco mutant could be rescued by complementation with a wild-type version of CrCO (SI Appendix, Fig. S6D), confirming that the phenotype is caused by the crco deletion.

To determine if CrCO is a downstream target of the putative COP1-SPA1 E3 ubiquitin ligase complex in C. reinhardtii, crco was crossed with spa1-1 to generate a crco spa1-1 double mutant. The double mutant had the same low NPQ phenotype as the crco single mutant (Fig. 4A and B), implying that SPA1 and CrCO act in the same pathway with CrCO downstream of SPA1. Because A. thaliana CO is a transcriptional regulator, we next examined whether transcript levels of the genes necessary for qE were affected in these mutants. Specifically, we measured transcript abundance for LHCSR1, LHCSR3.1, LHCSR3.2, PSBS1, and PSBS2 in 4 genotypes: WT, spa1-1, crco, and crco spa1-1. In spa1-1, each qE gene was expressed in LL at levels comparable to WT in HL (Fig. 4C), showing that spa1-1 disrupts the normal light-dependent expression of qE genes at the RNA level. The crco and crco spa1-1 mutants showed severely diminished expression of all 5 qE genes in both LL and HL, although some degree of HL induction persists (Fig. 4C). In fact, for LHCSR3.1 and LHCSR3.2, while crco and crco spa1-1 share the same diminished transcript abundance relative to WT for all 5 qE genes, the HL-induced fold induction at specifically these 2 genes remains robust.

At the protein level, the overaccumulation of LHCSR1 and PSBS observed in spa1-1 in HL (Fig. 4C and D and SI Appendix, Fig. S1) is correlated with their elevated transcript abundance. Similarly, the elevated transcript level of LHCSR1 in LL is associated with high LHCSR1 protein levels in LL (Fig. 4C and D); however, this was not the case for either LHCSR3 or PSBS in LL. LHCSR3 in LL-grown spa1-1 was detected at a similar level to WT LL (Fig. 4D), despite a large overaccumulation of LHCSR1 and LHCSR3.2 transcripts in spa1-1 LL (Fig. 4C). A similar trend emerged for PSBS. We observed a high accumulation of PSBS in spa1-1 HL and a relatively low level in WT HL. However, the levels of PSBS1 and PSBS2 transcripts in spa1-1 LL were equal to or greater than, respectively, their levels in WT HL, and yet we were unable to detect PSBS protein in spa1-1 LL. These results
target CrCO for proteolysis in LL, and the loss of either complex is sufficient to allow accumulation of CrCO protein. Alternatively, the DET1 complex and the COP1-SPA1 complex may target different transcription factors, and the aberrant accumulation of either transcription factor licenses expression of the qE genes. These models are not mutually exclusive, and because det1 mutants appear to have entirely lost light-intensity-dependent control of qE gene expression (38), DET1 might participate in multiple pathways governing photoprotection genes, perhaps including both the SPA1/CrCO-dependent and -independent pathways depicted in Fig. 5.

Our work has directly focused on the downstream components of this signaling cascade and thus, it is interesting to speculate on what might be upstream of SPA1 in mediating light regulation of the qE genes. In plants, COP1-SPA functions in phytochrome-mediated light signaling (40, 41); however in C. reinhardtii,
phytochrome proteins are absent (19). Thus, it seems likely that in combination with the different downstream wiring of CrCO targets in C. reinhardtii, the upstream control of the COP1-SPA1 E3 ubiquitin ligase complex has also diverged. One candidate for an upstream photoreceptor controlling COP1-SPA1 activity in C. reinhardtii is the UV-B photoreceptor UVRS, which is known to control LHCSR1 and PSBS accumulation in Chlamydomonas (18, 21). In A. thaliana, UVRS signaling converts COP1 from a repressor of photomorphogenesis into an activator through direct interaction with COP1 (42). It is conceivable that in spa1-1, mutation of a major COP1-interacting partner facilitates the interaction of UVRS and COP1; however, UVRS signaling in A. thaliana is not affected by loss of SPA proteins (43). C. reinhardtii SPA1 and its plant- and animal-type cryptochromes, and cryptochromes are known to interact with SPA proteins in A. thaliana (44–46). A C. reinhardtii insertional mutant that is defective in the animal-type cryptochrome exhibits altered light-dependent expression of several genes, including CrCO (47), and a mutant that diminishes abundance of the plant-type cryptochrome was shown to have impaired circadian clock control (48). However, HL induction of qE capacity and LHCSR3 accumulation is unaffected in the mutant affecting the animal-type cryptochrome (20). In contrast, the importance of PHOT in the control of photoprotection in C. reinhardtii has been clearly established through analysis of a phot mutant (20). We note that the phenotypes of the phot and creo mutants are similar, which suggests that PHOT and CrCO might act in the same pathway. In the phot mutant, as in creo (Fig. 4C), LHCSR1, LHCSR3.1, LHCSR3.2, PSBS1, and PSBS2 transcript levels are only a fraction of WT levels in HL and yet some degree of HL induction of gene expression persists at these genes (20, 38). Induction of qE gene expression by the PHOT pathway, however, also depends on a chloroplast-derived HL signal (20), unlike the SPA1/CrCO-dependent pathway shown in Fig. 5.

In addition, it has not escaped our notice that the chlorophyte SPA1 has a canonical CheY-like response regulator domain at its N terminus (Fig. 2B), which is absent in plant orthologs of SPA1, suggesting that a sensor histidine kinase may regulate SPA1 activity in C. reinhardtii. The presence of this response regulator domain in C. reinhardtii SPA1 might provide a clue about how the upstream regulation of the SPA1-COP1 E3 ubiquitin ligase complex has diverged from that described in A. thaliana. Although we have not yet characterized any point mutations in the response regulator domain of SPA1, dozens of suppressor mutants obtained in our UV mutagenesis of npq4 remain to be sequenced.

Fig. 5. Model depicting how the COP1-SPA1 E3 ubiquitin ligase complex in C. reinhardtii controls the light regulation of qE gene expression through the transcriptional regulator CrCO. In low light, ubiquitination of CrCO by the COP1-SPA1 E3 ubiquitin ligase targets CrCO for degradation. The low level of CrCO that persists in low light activates low levels of transcription from the qE genes (LHCSR1, LHCSR3.1, LHCSR3.2, PSBS1, and PSBS2). In high light, a photoreceptor-mediated signal inhibits the COP1-SPA1 E3 ubiquitin ligase by an undefined mechanism, allowing accumulation of CrCO and its binding to the CO-like binding motif and transcriptional activation of the qE gene promoters. In addition, to explain the high-light induction of qE gene expression in the creo and creo spa1-1 mutants (Fig. 4 C and D) we hypothesize the existence of a second transcriptional regulator bound to the qE gene promoters that mediates a SPA1/CrCO-independent but high-light-dependent regulatory pathway. Whether this pathway constitutes a second photoreceptor-mediated pathway, possibly integrated with a chloroplast retrograde signaling pathway, is unknown.

Materials and Methods

Strains, Growth Conditions, and UV Mutagenesis. C. reinhardtii wild-type strain 4A+ (mt+, 137c background; CC-4051), npq4 mutant defective in LHCSR3.1 and LHCSR3.2 genes (6), creo (CAL028 01 08) insertion mutant (37), and npq4 suppressor mutants were grown in minimal high-salt medium (49) in continuous light at 60 μmol photons m−2 s−1 (LL) and 25°C for 3 to 3 d to a cell density of 3 to 4 × 106 cells mL−1, and then cells were either maintained at the same light intensity or transferred to 350 μmol photons m−2 s−1 (HL) for the specified time before harvesting cells for various measurements. Unless otherwise specified, 3 biological replicates were used. The spa1 and cul4 mutants were generated by UV mutagenesis of npq4 with 6 or 7 × 1012 μm−2 cm−2 of UV light (Stratagene, La Jolla, CA) as previously described (50).

Chlorophyll Fluorescence Measurements. Chlorophyll fluorescence was measured at room temperature using a pulse-amplitude-modulated fluorometer (FMS2; Hansatech Instruments) or an Imaging-PAM Maxi (Walz). Cells were dark acclimated for 30 min prior to measurement, unless stated otherwise. Maximum fluorescence levels after dark acclimation (Fm) and maximum fluorescence levels in the light (Fm′) were recorded after applying a saturating pulse of light. NPQ was calculated as (Fm′ − Fm)/Fm′. For the FMS2 measurements, 3 to 5 × 104 cells were filtered on a glass-fiber filter that was placed in the instrument’s leaf clip. This was followed by an additional 5-min dark phase with far-red light of 0.6 μmol photons m−2 s−1. Fm′ and Fm were recorded after applying a saturating pulse of 1,800 μmol photons m−2 s−1. NPQ was induced for 9.5 min with 600 μmol photons m−2 s−1, followed by recovery in the dark for far-red light. On the Imaging-PAM, NPQ was induced for 6 to 10 min with 550 μmol photons m−2 s−1 and relaxed for 2.5 to 8 min in the dark. False-colored NPQ images were generated by the Walz software.

Bulked Segregant Analysis and Genome Sequencing. Genetic crosses and tetrad analysis were performed according to established methods (49). The npq4 suppressor strains were backcrossed to the npq4 strain and selfed. Tetrads were dissected, and the NPQ phenotype of all progeny was scored using the Imaging-PAM Maxi. Bulked segregant pools for spa1-1 and cul4-1 were prepared by combining the progeny from each tetrad into 2 separate pools (1 with all high-NPQ progeny and 1 with all low-NPQ progeny) for genomic DNA preparation and whole-genome sequencing. The progeny originated from 7 complete tetrads for both spa1-1 and cul4-1. The library preparation and sequencing (illumina HiSeq4000) of the spa1-1 and cul4-1 bulked segregant pools was performed at the Vincent J. Coates Genomics Sequencing Laboratory at University of California (UC), Berkeley. Sequencing reads were filtered and trimmed using Trimmomatic (51), cutadapt (52), and Super-Deduper (53). Overlapping paired reads were merged using Flash (54). Cleaned sequence data (paired, merged, and orphan reads) from each individual sample were then aligned to the reference genome (Chlamydomonas reinhardtii v5.5) using Novoalign (Novocept Technologies). Only reads that mapped uniquely to the reference were kept. After alignment, we used Picard (http://broadinstitute.github.io/picard) to add read groups and GATK (55) to perform realignment. We used GATK HaplotypeCaller to determine SNP and short indel variants from each individual with the aim of retaining all potential variants. For the resequencing of npq4, spa1-1, spa1-2, and cul4-1, the same sequencing and bioinformatic pipeline tools were used. The final coverage for the resequenced samples and the bulked segregant pools (spa1-1 high NPQ progeny, spa1-1 low NPQ progeny, cul4-1 high NPQ progeny, cul4-1 low NPQ progeny) was ~100×. For spa1-1, 3 filtered variants in Cre13.gS91050, Cre13.gS91150, and
Cre13.g602700 (SPA1) had an alternate allele frequency of 100% in the high NPQ pool vs. 0% in the low NPQ pool, each with a minimum coverage of 149×. Three variants (Table S5). For crco-1, the maximum alternative allele frequency among the filtered variants was 99.6% in the high NPQ pool vs. 0.01% in the low NPQ pool, and this variant was located in Cre12.g516500 (CUL4) with a minimum coverage of 197× (SI Appendix, Table S2). The mutation in spa-1 was identified by resequencing; Cre13.g602700 (SPA1) was the only gene that contained mutations in both spa-1 and spa-2.

**Complementation of npq4 spa-1 and crco spa-1.** To complement npq4 spa-1, an 8.5-kb fragment was amplified by PCR with KOD hot start DNA polymerase (Millipore) and primers gSPA1-F and gSPA1-R (SI Appendix, Table S3) from a BAC clone (3M23) containing C. reinhardtii genomic DNA of this region. The 8.5-kb fragment contains 981 bp upstream of the start codon, the SPA1 gene, and the endogenous 3′–UTR. The PCR product was cloned using the Zero Blunt TOPO PCR Cloning kit (Thermo Fisher Scientific) and confirmed by sequencing. The npq4 spa-1 strain was cotransformed by electroporation with the SPA1 plasmid and linearized pSL18 (56) that carries paromomycin resistance. Out of 150 transformants screened, 11 showed high NPQ like that of npq4. As expected, none of the 350 transformants obtained with only the pSL18 vector showed a low level of NPQ.

**Gene Expression Analysis Using Real-Time qPCR.** RNA extraction, cDNA synthesis, and qPCR were performed as previously described (62). All primer pairs (SI Appendix, Table S3) were confirmed as having 90 to 105% amplification efficiency and linear amplification within their dynamic range in experimental samples using serial dilutions of cDNA prior to the experiments. Relative transcript levels were calculated by ∆ΔCT method (63) using CPlast as the internal reference. Primers were designed using Primer3 (64) against the 3′-UTR of each gene to avoid binding to off-target paralogous genes. A single peak in melt curve analysis with a unique melting temperature was observed for each amplicon, verifying that off-target amplification of paralogous genes was negligible.

**Motif Analysis.** The position-specific scoring matrix for the CO-like binding site upstream of the C. reinhardtii npq genes was identified de novo by performing Multiple Em for Motif Elucidation (MEME) (32). To increase the number of sequences submitted to MEME, orthologous promoter sequences from Volvox carteri were included.

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29. D. Zhu et al., Biochemical characterization of Arabidopsis complexes containing CONSTITUTIVELY PHOTOMORPHOGENIC1 and SUPPRESSOR OF PHYA proteins in light control of plant development. Plant Cell 20, 2307–2323 (2008).

30. X. Holtkotte et al., Mutations in the N-terminal kinase-like domain of the represor of photomorphogenesis SPA1 severely impair SPA1 function but not light responsiveness in Arabidopsis. Plant J. 88, 205–216 (2016).

31. S. Chen et al., The functional divergence between SPA1 and SPA2 in Arabidopsis photomorphogenesis maps primarily to the respective N-terminal kinase-like domain. BMC Plant Biol. 16, 165 (2016).

32. T. L. Bailey et al., MEME SUITE: Tools for motif discovery and searching. Nucleic Acids Res. 37, W202–W208 (2009).

33. N. Gnesutta et al., CONSTANS imparts DNA sequence specificity to the histone fold NF-YB/NF-TC dimer. Plant Cell 29, 1516–1532 (2017).

34. S. Laubinger et al., Arabidopsis SPA proteins regulate photoperiodic flowering and interact with the floral inducer CONSTANS to regulate its stability. Development 133, 3213–3222 (2006).

35. L.-J. Liu et al., COP1-mediated ubiquitination of CONSTANS is implicated in cryptochrome regulation of flowering in Arabidopsis. Plant Cell 20, 292–306 (2008).

36. S. Jang et al., Arabidopsis COP1 shapes the temporal pattern of CO accumulation conferring a photoperiodic flowering response. EMBO J. 27, 1277–1288 (2008).

37. R. M. Dent et al., Large-scale insertional mutagenesis of Chlamydomonas supports phylogenomic functional prediction of photosynthetic genes and analysis of classical acetate-requiring mutants. Plant J. 82, 337–351 (2015).

38. Y. Aihara, K. Fujimura-Kamada, T. Yamasaki, J. Minagawa, Algal photoprotection is regulated by the E3 ligase CUL4-DDB1–COP1 and RED1 in the UV-B response in Chlamydomonas reinhardtii. Proc. Natl. Acad. Sci. U.S.A. 110, 16669–16674 (2013).

39. O. S. Lau, X. W. Deng, The photomorphogenic repressors COP1 and DET1: 20 years of research. Trends Plant Sci. 20, 1516–1525 (2015).

40. D. J. Sheerin et al., Light-activated phytochrome A and B interact with members of the SPA family to promote photomorphogenesis in Arabidopsis by reorganizing the COP1/SPA complex. Plant Cell 27, 189–201 (2015).

41. X.-D. Lu et al., Red-light-dependent interaction of phyB with SPA1 promotes COP1-SPA dissociation and photomorphogenic development in Arabidopsis. Mol. Plant 8, 467–478 (2015).

42. X. Huang et al., Conversion from CUL4-based COP1-SPA E3 apparatus to UVR8/COP1-SPA complexes underlies a distinct biochemical function of COP1 under UV-B. Proc. Natl. Acad. Sci. U.S.A. 110, 11669–11674 (2013).

43. A. Oravecz et al., CONSTITUTIVELY PHOTOMORPHOGENIC1 is required for the UV-B response in Arabidopsis. Plant Cell 18, 1975–1990 (2006).

44. B. Liu, Z. Guo, H. Liu, X. Liu, C. Lin, Arabidopsis cryptochrome 1 interacts with SPA1 to suppress COP1 activity in response to blue light. Genes Dev. 25, 1029–1034 (2011).

45. H.-L. Lian et al., Blue-light-dependent interaction of cryptochrome 1 with SPA1 defines a dynamic signaling mechanism. Genes Dev. 25, 1023–1028 (2011).

46. Z. Zuo, H. Liu, B. Liu, X. Liu, C. Lin, Blue light-dependent interaction of CRY2 with SPA1 regulates COP1 activity and floral initiation in Arabidopsis. Curr. Biol. 21, 841–847 (2011).

47. B. Beel et al., A flavin binding cryptochrome photoreceptor responds to both blue and red light in Chlamydomonas reinhardtii. Plant Cell 24, 2992–3008 (2012).

48. N. Müller et al., A plant cryptochrome controls key features of the Chlamydomonas circadian clock and its life cycle. Plant Physiol. 174, 185–201 (2017).

49. E. H. Harris, The Chlamydomonas Sourcebook (Academic Press, Inc., San Diego, 1989).

50. S. S. McCarthy, M. C. Kobayashi, K. K. Niyogi, White mutants of Chlamydomonas reinhardtii are defective in phytoene synthase. Genetics 168, 1249–1257 (2004).

51. A. M. Bolger, M. Lohse, B. Usadel, Trimomatic: A flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120 (2014).

52. M. Martin, Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J. 17, 10–12 (2011).

53. K. R. Petersen, D. A. Street, A. T. Gerritsen, S. S. Hunter, M. L. Settles, “Super deducer, fast PCR duplicate detection in fastq files” in Proceedings of the 6th ACM Conference on Bioinformatics, Computational Biology and Health Informatics (Association for Computing Machinery, New York, 2015), pp. 491–492.

54. T. Magot, S. L. Salzberg, FLASH: Fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27, 2957–2963 (2011).

55. A. McKenna et al., The genome analysis toolkit: A MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 20, 1297–1303 (2010).

56. N. Depège, S. Bellaïche, J.-D. Rochaix, Role of chloroplast protein kinase Stt7 in LHCII phosphorylation and state transition in Chlamydomonas. Science 299, 1572–1575 (2003).

57. P. Berthold, R. Schmitt, W. Mages, An engineered Streptomyces hygroscopicus aph 7* gene mediates dominant resistance against hygromycin B in Chlamydomonas reinhardtii. PloS One 153, 401–412 (2002).

58. R. H. Calderon et al., A conserved rubredoxin is necessary for photosystem II accumulation in diverse oxygenic photoautotrophs. J. Biol. Chem. 288, 26688–26696 (2013).

59. D. Wessel, U.-l. Flügge, A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal. Biochem. 138, 141–143 (1984).

60. U. K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685 (1970).

61. C. Richard, T. D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-ΔΔCt) method. Methods 25, 402–408 (2001).

62. A. Untergasser et al., Primer3–New capabilities and interfaces. Nucleic Acids Res. 40, e115 (2012).