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Small RNA-mediated silencing of phototropin suppresses the induction of photoprotection in the green alga *Chlamydomonas reinhardtii*

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Small RNAs (sRNAs) form complexes with Argonaute proteins and bind to transcripts with complementary sequences to repress gene expression. sRNA-mediated regulation is conserved in a diverse range of eukaryotes and is involved in the control of various physiological functions. sRNAs are present in the unicellular green alga *Chlamydomonas reinhardtii*, and genetic analyses revealed that the core sRNA biogenesis and action mechanisms are conserved with those of multicellular organisms. However, the roles of sRNAs in this organism remain largely unknown. Here, we report that *Chlamydomonas* sRNAs contribute to the induction of photoprotection. In this alga, photoprotection is mediated by LIGHT HARVESTING COMPLEX STRESS-RELATED 3 (LHCSR3), whose expression is induced by light signals through the blue-light receptor phototropin (PHOT). We demonstrate here that sRNA-defective mutants showed increased PHOT abundance leading to greater *LHCSR3* expression. Disruption of the precursor for two sRNAs predicted to bind to the *PHOT* transcript also increased PHOT accumulation and *LHCSR3* expression. The induction of *LHCSR3* in the mutants was enhanced by light containing blue wavelengths, but not by red light, indicating that the sRNAs regulate the degree of photoprotection via regulation of *PHOT* expression. Our results suggest that sRNAs are involved not only in the regulation of photoprotection but also in biological phenomena regulated by PHOT signaling.

Chlamydomonas | microRNA | photoprotection

Significance

Small RNAs (sRNAs) are important noncoding RNAs that act on mRNAs harboring complementary sequences to control gene expression and thereby regulate various physiological phenomena in multicellular organisms. This mechanism is widely present in multicellular eukaryotes and is thought to have been established in the last common ancestor of animals and plants, which was a unicellular organism, but its role in extant unicellular eukaryotes has not been well studied. In this study, we determined that sRNAs regulate the abundance of the blue-light receptor phototropin in the unicellular green alga *Chlamydomonas reinhardtii* and are involved in the regulation of a photoprotection mechanism of the photosynthetic system against excess light, which is driven by blue-light signaling.

Small RNAs (sRNAs), which include microRNAs (miRNAs) and small interfering RNAs in animals and plants, are short RNA molecules, 20 to 24 nucleotides in length, that usually function as negative regulators of gene expression by binding to complementary sequences in their target transcripts, causing posttranscriptional silencing through the cleavage and/or translation inhibition of target RNAs (1–3).

The sRNA-mediated gene silencing system is evolutionarily conserved in diverse eukaryotic species, suggesting that the pathway was present in the last common ancestor of plants and animals (4–6). In general, sRNA biogenesis begins with the cleavage, via a microprocessor complex composed of RNase III enzymes such as Dicer and double-stranded RNA-binding proteins (dsRBPs), of incomplete or nearly complete stem loops in the precursor RNA that is transcribed from the inverted repeat region of genomic sRNA loci. One strand of the double-stranded sRNA is incorporated into the effector protein ARGONAUTE (AGO) to form the RNA-induced silencing complex (RISC), which uses the sRNA as a guide strand to bind to mRNAs with complementary sequences. The high complementarity of sRNA–mRNA hybrids seen in plants often induces endonucleolytic cleavage of the transcript, while incomplete hybrids more common in animals often induce translational repression and cleavage-independent destabilization of the target transcript. sRNAs regulate biological phenomena such as developmental timing, cell proliferation, and cell death in animals and plants (1–3). The physiological roles of sRNAs have been clarified exclusively in multicellular model organisms, but little is known about which genes are regulated by sRNAs in unicellular eukaryotes or their physiological roles.

The first unicellular organism reported to have canonical miRNAs was the green alga *Chlamydomonas* (*Chlamydomonas reinhardtii*) (7, 8). Although *Chlamydomonas* is evolutionarily distant from animals and land plants, a genetic analysis of sRNA-mediated silencing-defective mutants demonstrated the conservation of the core mechanisms of canonical sRNA biosynthesis and their repressive actions on target mRNAs, indicating that this posttranscriptional regulatory mechanism is fully functional in this alga (9). For instance, the *Chlamydomonas* microprocessor complex is composed of DICER-LIKE 3 (DCL3) and DULL SLICER 16 (DUS16, a dsRBP), and loss of function of either protein significantly decreases the abundance of most types of miRNA. AGO3 is the only AGO

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The authors declare no competing interest.

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among AGO1–3 that is present in the cytoplasm and forms a RISC with miRNAs to repress gene expression. Thus, the miRNA-mediated silencing of target gene expression was almost lost in mutants lacking *DCL3*, *DUS16*, or *AGO3* (10–14).

Despite the identification of many novel sRNAs in *Chlamydomonas* grown under various culture conditions, their target genes have rarely been experimentally confirmed; therefore, their biological roles are largely unknown (15–17). One reason for the lack of progress in elucidating the physiological role of sRNAs in *Chlamydomonas* is that mutants in *AGO3*, *DCL3*, or *DUS16* do not show a lower growth rate or obvious cellular abnormalities (10, 11, 14). Another reason may be that the mode of sRNA-mediated silencing in *Chlamydomonas* is mainly via translational repression of target transcripts, which might cause only subtle changes in target transcript levels, making it difficult to identify the target genes of the sRNAs in a transcriptome deep sequencing (RNA-Seq) analysis of the mutants (18). Without a protein-level analysis, it is difficult to distinguish whether a candidate target gene predicted from sRNA–mRNA homology is translationally repressed (19).

Chlamydomonas is a photosynthetic organism; light is essential for photosynthesis and its survival in the absence of a reduced carbon source, but excess light intensity beyond its assimilative capacity can cause oxidative stress and even cell death (20, 21). As a preventive mechanism against such unwanted effects, plants and microalgae are equipped with a photoprotective mechanism, qE, which dissipates excess light energy as thermal energy. In *Chlamydomonas*, qE is mediated by the photoprotective protein PHOTOSYSTEM II SUBUNIT S (PSBS) and two LIGHT HARVESTING COMPLEX STRESS-RELATED (LHCSR) proteins, LHCSR1 and LHCSR3 (22). *LHCSR3* expression is initiated by a wavelength-specific light signaling pathway through the blue-light photoreceptor phototropin (PHOT) (21). PHOT is a blue light–activated kinase that regulates various responses in land plants, including phototropism, stomatal opening, and chloroplast movement (23–26). In *Chlamydomonas*, PHOT is triggered by blue light and plays a role in the production of various pigments (27), as well as proteins that protect against light stress (21, 28). Here, we report that sRNAs repress the accumulation of PHOT, thereby reducing the sensitivity of the alga to blue light and repressing the expression of *LHCSR3*. We propose that *Chlamydomonas* sRNAs play a role in optimizing photoprotective activity by reducing the extent of *LHCSR3* induction by blue light via repression of *PHOT* expression.

Results

PHOT Is More Abundant and *LHCSR3* Expression Is Enhanced in sRNA-Defective Mutants. We performed a simple immunoblot analysis on the sRNA-defective mutant *dus16-1* (10) grown under various conditions using available antibodies, with the aim of identifying proteins that accumulated to higher levels in the mutant and, from there, potential target gene candidates. We determined that the blue light–dependent induction of LHCSR3 accumulation was slightly enhanced in *dus16-1* compared to its parental strain Gluc(1×) under low blue light (~20 μmol/m^2/s) but not under high blue light (~250 μmol/m^2/s) (Fig. 1*A*). LHCSR3 functions by binding to photosystem II in the chloroplast to induce qE (29). Since *LHCSR3* expression is induced by blue-light signaling via PHOT (21), we focused on PHOT and established that PHOT abundance was also increased in *dus16-1* but returned to levels similar to that seen in Gluc(1×) in its complemented strains (Fig. 1*B*). However, *PHOT* transcript levels were not significantly altered (Fig. 1*C*). Accordingly, we observed enhanced expression of *LHCSR3* in *dus16-1* relative to the parental strain under blue light or white light but not under red light (Fig. 1 *B*

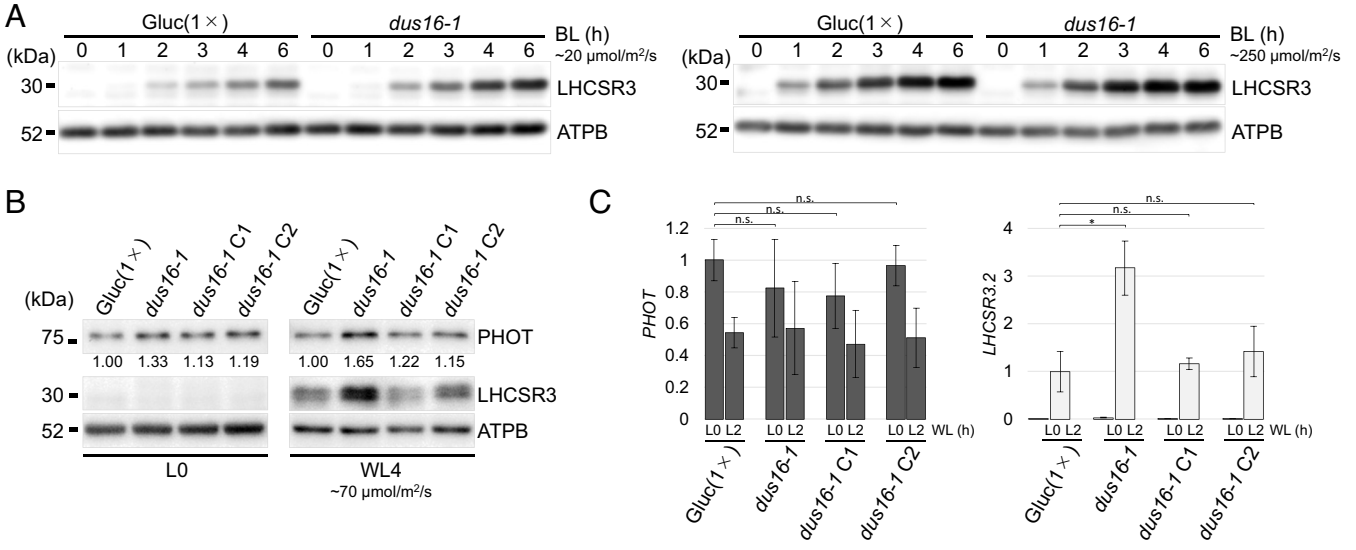

Fig. 1. PHOT is increased and *LHCSR3* expression is enhanced in the sRNA-defective mutant *dus16-1*. (*A*) Immunoblot analysis of LHCSR3. Gluc(1×) harbors a *Gaussia* luciferase transgene designed to be cleaved by miR9897-3p in the CC-124 background; *dus16-1, DUS16* insertion mutant generated by random insertional mutagenesis of Gluc(1×). Gluc(1×) and *dus16-1* were precultured photoautotrophically under continuous white light (~20 μmol/m^2/s) from LEDs and irradiated with different intensities of blue LED light (BL, ~20 or ~250 μmol/m^2/s) for LHCSR3 induction. Protein samples were collected at the indicated time points and used for analysis. ATPB served as loading control. (*B*) Immunoblot analysis of PHOT and LHCSR3. *dus16-1* C1 and C2, complementation strains expressing FLAG-HA-tagged *DUS16* in the *dus16-1* background. The indicated strains were precultured photoautotrophically in a photoperiod of 12 h under ~40 μmol/m^2/s white LED light and 12 h in the dark for synchronization. At the end of the dark period (L0), the cells were exposed to white LED light at ~70 μmol/m^2/s for 4 h (WL4). PHOT and ATPB protein levels were quantified from signal intensity, and PHOT abundance was normalized to that of ATPB, with the values from Gluc(1×) set to 1. (*C*) RT-qPCR analysis of *PHOT* and *LHCSR3*. Total RNAs were purified from cells collected at the end of the dark period (L0) and after 2 h of white LED light exposure (L2) as in (*B*). *PHOT* and *LHCSR3.2* transcript levels were normalized to the levels of *NUOP4*, an NADH:ubiquinone oxidoreductase gene whose expression level is constant throughout the cell cycle (30). The value corresponding to L0 of Gluc(1×) for *PHOT* levels was set to 1, the value corresponding to L2 of Gluc(1×) for *LHCSR3.2* was set to 1, and other values were adjusted accordingly. The values shown are means ± SD of three independent biological replicates. Two-tailed, unpaired *t* test was used to reveal statistically significant difference. *$P \le 0.05$ and n.s., not significant.

and *C* and *SI Appendix*, Fig. S1). Moreover, we detected similar differences in the levels of PHOT protein and in the LHCSR3 accumulations in response to different light signals in *ago3-1*, a mutant defective in AGO3 function (*SI Appendix*, Fig. S1). These results suggest that one or more sRNA(s) can repress PHOT translation from its transcript and indirectly enhance the induction of *LHCSR3* expression.

Multiple Target Sequences in the *PHOT* Transcript Can Be Recognized by sRNAs. To screen for *Chlamydomonas* sRNAs that potentially repress *PHOT* expression, we performed a psRNAtarget analysis (31) with 291 sRNA sequences [named *Chlamydomonas reinhardtii* sRNA (crs)1–291; *SI Appendix*, Table S1] that had counts of more than 20 per million reads in a previous sRNA-Seq analysis of the Gluc(1×) strain (11). We predicted that 21 sRNAs can bind to the *PHOT* transcript (*SI Appendix*, Table S2). Metazoan miRNAs, whose recognition rules and modes of silencing for their target mRNAs are similar to those in *Chlamydomonas*, can induce the translational repression of their targets when accumulating to high enough levels (32). We therefore hypothesized that high sRNA abundance was required to promote repression of target genes in *Chlamydomonas*. We therefore focused on two abundant (more than 200 counts per million reads) sRNAs: crs13 [previously annotated as miR910 (7, 8) and referred to as miR910 hereafter] and crs49 (*SI Appendix*, Table S2 and Figs. S2–S4). crs49 was one of the 10 (crs49, crs164, crs169, crs176, crs178, crs262, crs266, crs276, crs279, and crs288) of the 21 sRNAs potentially binding to *PHOT* transcripts that were derived from a single precursor transcript of Cre13.g605300 (*SI Appendix*, Fig. S3). Among these 10 sRNAs, crs49 and its variants (crs169, crs176, crs276, crs288) were highly abundant and thus most likely to predominantly contribute to *PHOT* repression. miR910 and a group of sRNAs derived from Cre13.g605300 were substantially less abundant in *dus16-1* than in the parental strain (*SI Appendix*, Figs. S2 and S3) and are predicted to recognize six binding sites along the *PHOT* transcript (*SI Appendix*, Fig. S4), suggesting that these sRNAs might synergistically repress *PHOT* expression.

Lower miR910 and crs49 Abundance Results in Increased PHOT Levels, Enhanced *LHCSR3* Expression, and a Concomitant Increase in Non-photochemical Quenching (NPQ). According to strict criteria for plant miRNA annotation (33), miR910 and crs49 cannot be categorized as miRNAs, but only as sRNAs. Nevertheless, they are embedded in a hairpin-shaped precursor and their abundance was lower in *dus16-1* (*SI Appendix*, Figs. S2 and S3), indicating that they are generated through the typical miRNA biogenesis pathway and probably possess gene silencing activity. It was not clear which sRNA contributed to the repression of *PHOT* expression, as the abundance of most sRNA species was lower in *dus16-1* relative to the parental strain. To identify the responsible sRNA, we separately disrupted the inverted repeats located within Cre14.g629200 and Cre13.g605300 using CRISPR, CRISPR-Cas/CRISPR-associated protein 9 (Cas9)-mediated genome editing (*SI Appendix*, Fig. S5). We subjected the isolated genome-edited strains to four rounds of backcrossing to the wild-type strain before analyses; these mutants are designated *mir910-1* and *crs49-1*, respectively. The insertion of donor DNA into the seventh intron of Cre14.g629200 in *mir910-1* did not alter the miR910 sequence (*SI Appendix*, Fig. S5) but may have caused changes in the precursor structure (*SI Appendix*, Fig. S6). This structural change presumably perturbs accurate recognition of the precursor and subsequent processing by the microprocessor, such that the accumulation of mature miR910 is lower than that in wild-type cells (Fig. 2*A*). Conversely, the insertion of

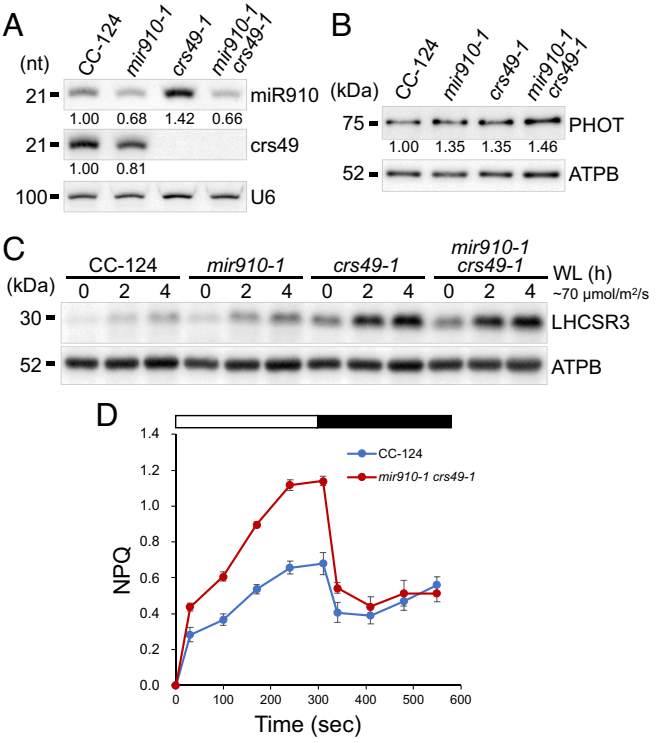

Fig. 2. PHOT is increased, the induction of *LHCSR3* expression is enhanced, and NPQ is enhanced in the *miR910-1 crs49-1* double mutant. (*A*) Northern blot analysis of miR910 and crs49 in CC-124 and the genome-edited mutants. *Mir910-1*, CRISPR/Cas9-mediated genome-edited mutant of the *MIR910* precursor region within Cre14.g629200 in the CC-124 background; *crs49-1*, CRISPR/Cas9-mediated genome-edited mutant of the *CRS49* precursor region within Cre13.g605300 in the CC-124 background; *mir910-1 crs49-1*, double mutant generated by a cross between *mir910-1* and *crs49-1*. U6 small nuclear RNA was used as loading control. The levels of sRNAs were normalized to those of U6 and compared to those in CC-124. The same membranes were used to detect miR910, crs49, and U6, in that order. (*B*) Immunoblot analysis of PHOT in the indicated strains. The strains were cultured photoautotrophically under continuous white LED light (~20 µmol/m^2/s) and their total proteins were sampled. (*C*) Immunoblot analysis of LHCSR3 in the indicated strains. Cells were precultured photoautotrophically under continuous white LED light (~20 µmol/m^2/s) and irradiated with additional white LED light (~70 µmol/m^2/s) to induce *LHCSR3* expression. (*D*) NPQ analysis of chlorophyll fluorescence in CC-124 and *mir910-1 crs49-1*. Cells were cultured photoautotrophically under continuous white LED light (~30 µmol/m^2/s) and used for NPQ analysis. The values shown are means ± SD of three independent replicates.

donor DNA in *crs49-1* is predicted to split the crs49* sequence (*SI Appendix*, Fig. S5), preventing production of the crs49/crs49* duplex (*SI Appendix*, Fig. S7). Accordingly, we did not detect crs49 (Fig. 2*A*). In addition, we detected a slight decrease in crs49 abundance in *mir910-1*, as well as some increase in miR910 levels in *crs49-1*, suggestive of a reciprocal regulation of their abundance (Fig. 2*A*). The two genome-edited strains showed a slight increase in PHOT abundance and enhanced LHCSR3 accumulation compared to the parental strain, CC-124 (Fig. 2*B*); PHOT and LHCSR3 abundance declined in the complemented strains expressing artificial miR910 or crs49 (*SI Appendix*, Fig. S8). Moreover, the double mutant (*mir910-1 crs49-1*, generated from a cross between *mir910-1* and *crs49-1*) displayed an even greater increase in PHOT protein levels (Fig. 2*B*), suggesting that sRNAs, including at least miR910 and crs49, repress *PHOT* expression. We also confirmed that other sRNAs showed limited changes in abundance in the single and double mutants: miR912, miR1157, miR1162, miR1169, and miR9897-3p (*SI Appendix*, Fig. S9).

We next investigated the effect on *LHCSR3* induction in algal cells lacking specific sRNAs. Following irradiation with white

light, the *mir910-1* mutant accumulated only slightly more LHCSR3 protein, while *crs49-1* accumulated much more LHCSR3 protein than the wild-type strain CC-124 (Fig. 2*C*). The degree of *LHCSR3* induction in the *mir910-1 crs49-1* double mutant was comparable to that in *crs49-1* (Fig. 2*B*). In addition to increased *LHCSR3* expression, we detected LHCSR3 accumulation in *crs49-1* and *mir910-1 crs49-1* under preculture conditions with a light intensity that was insufficient to raise LHCSR3 abundance in CC-124 (Fig. 2*C*). This observation suggested that the loss of crs49 lowers the light intensity threshold required for LHCSR3 induction. Higher levels of LHCSR3 are suggestive of enhanced photoprotection; accordingly, we measured the NPQ of chlorophyll fluorescence, which was about twice as high in the double mutant than that in CC-124 (Fig. 2*D*). These results suggest that crs49 and possibly miR910 also contribute to repressing *PHOT* expression, thereby reducing the sensitivity of *LHCSR3* induction to low light.

Enhancement of *LHCSR3* Induction in *mir910-1 crs49-1* Depends on a Blue-Light Signal. In wild-type cells, *LHCSR3* is efficiently induced by weak blue light but can also be induced by strong red light independently of PHOT (21). To test whether the observed increase for LHCSR3 in *mir910-1 crs49-1* was due to an increase in PHOT protein levels, we tested *LHCSR3* induction by white, blue, or red light starting at the end of the dark period in synchronously cultured cells, which were used because the efficiency of photoprotection induction is closely related to photoperiodic signaling in *Chlamydomonas* (34). We set the fluence of each light condition to a level that would barely induce *LHCSR3* in the double mutant to show the difference between this line and CC-124. Consistent with the above results, *LHCSR3* expression was strongly induced in the double mutant but not in CC-124 under low white-light treatment (Fig. 3). Similarly, *LHCSR3* was more strongly induced by blue light in the double mutant than that in CC-124 (Fig. 3). By contrast, we observed no obvious differences in LHCSR3 protein levels between the two genotypes under high white-light or red-light treatment, although slightly more *LHCSR3* transcripts accumulated under red light in the double mutant than that in CC-124 (Fig. 3). These results suggest that the increased amount of PHOT protein detected in the double mutant contributes to the enhanced induction of *LHCSR3* expression. Furthermore, PHOT protein levels tended to increase gradually with the time passed from the start of light exposure, except high white light, and this increase was slightly greater in the double mutant (Fig. 3). PHOT protein abundance increased in a light-responsive manner, but *PHOT* mRNA levels decreased substantially under the same conditions, indicating that *PHOT* mRNA and PHOT protein levels may be negatively correlated. Although miR910 or crs49 levels did not change substantially in CC-124 after light irradiation (*SI Appendix*, Fig. S10), the levels of miR910 and crs49 relative to *PHOT* mRNA levels increased. This suggests that the effect of both sRNAs on suppressing *PHOT* increased after light irradiation.

Discussion

Although sRNA-defective mutants of *Chlamydomonas* show no obvious phenotypic abnormalities (10, 11, 14), a physiological role for some sRNAs has been proposed in this organism. For example, miR914 was suggested to contribute to ultraviolet-B light resistance via the repression of *RIBOSOMAL PROTEIN L18* (*RPL18*), miR1166.1 to hydrogen production, and miR906-3p and miR910 to several abiotic stress adaptations (35–37). However, these suggestions have been inferred from studies in which each miRNA was overexpressed as an artificial miRNA and thus could not reveal which endogenous miRNAs regulate which target genes to alter the phenotype. Furthermore, the possibility of off-target effects due to overexpression could not be ruled out.

Other studies using sRNA-defective mutants have attempted to elucidate the molecular mechanisms by which sRNAs regulate

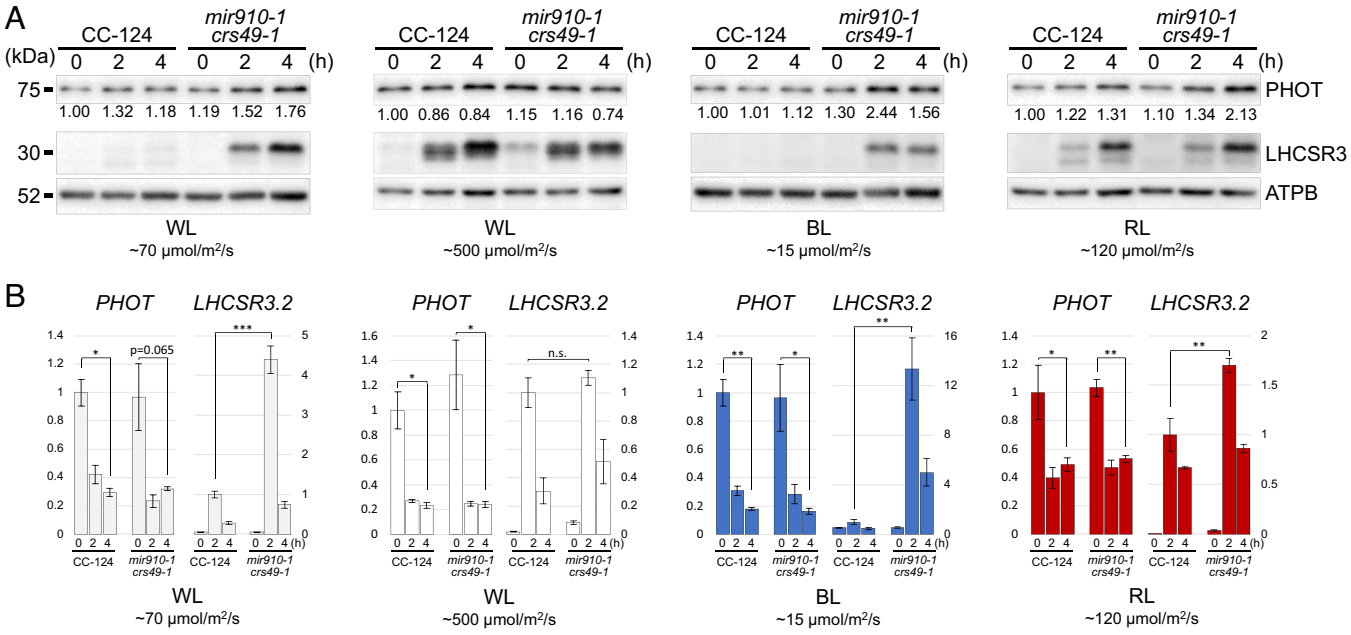

Fig. 3. Blue light–dependent enhancement of *LHCSR3* induction in *mir910-1 crs49-1*. The indicated strains were precultured photoautotrophically in a photoperiod of 12 h under ~40 µmol/m^2/s white LED light and 12 h in the dark for synchronization. At the end of the dark period, the cells were exposed to white LED light at ~70 µmol/m^2/s, white LED light at ~500 µmol/m^2/s, blue LED light at ~15 µmol/m^2/s, or red LED light (RL) at ~120 µmol/m^2/s. (*A*) Immunoblot analyses of PHOT and LHCSR3. (*B*) RT-qPCR analysis of *PHOT* and *LHCSR3.2*. The value corresponding to L0 of CC-124 for *PHOT* levels was set to 1, the value corresponding to L2 of CC-124 for *LHCSR3.2* was set to 1, and other values were adjusted accordingly. The values shown are means ± SD of three independent biological replicates. Two-tailed, paired (*PHOT*), and unpaired (*LHCSR3.2*) *t* test was used to reveal statistically significant difference. *$P \le 0.01$, **$P \le 0.01$, ***$P \le 0.001$ and n.s., not significant.

physiological functions, based on the functional analysis of genes whose transcripts or encoded proteins showed altered levels in the mutants. The genes Cre16.g683650 and Cre17.g697550 are silenced by miRNAs, but their role in regulating physiological functions is unknown (19, 38). In addition, several target transcripts were predicted to be candidates for translational repression from a comprehensive analysis using a combination of RNA-Seq and ribosome profiling (measuring actively translated transcripts), but how the repression of each affects the phenotype has not yet been analyzed (18).

In this report, we showed that the sRNAs crs49 and miR910 repressed *PHOT* expression, which attenuated the induction of *LHCSR3* expression and reduced the photoprotective capability of the alga *Chlamydomonas*. This case is an elucidation in *Chlamydomonas* of the cascade through which sRNAs regulate their target genes to control particular physiological functions, demonstrating the physiological significance of sRNAs in unicellular organisms. Our discovery was based on the incidental observation of a subtle increase in LHCSR3 protein levels in *dus16-1*, however, and it remains difficult to elucidate the role of sRNA functions without the elucidation of phenotypic abnormalities and/or target genes that must have their expression repressed by sRNAs. It was previously proposed that the primary mode of action of *Chlamydomonas* sRNAs on their target mRNAs was translational inhibition (18), and consistent with that hypothesis, PHOT translation appears to be repressed. We attempted to clone the truncated end of the *PHOT* transcript using 5′ rapid amplification of cDNA ends (5′ RACE) to test for sRNA-mediated cleavage. On one occasion, we were able to clone a truncated end that was one base off position 10 to 11 in miR910 (*SI Appendix*, Fig. S11). However, we failed to reproduce this result; none of the other truncated ends corresponding to other predicted cleavage sites were detected. The number of ribosomes on the target transcript does not change much because *Chlamydomonas* sRNA inhibits the translation reaction after initiation (18, 38, 39), suggesting that new approaches are required to comprehensively identify target mRNAs, such as cross-linking immunoprecipitation sequencing (40).

In *Chlamydomonas*, many of the stem-loops in which sRNAs are embedded are located in introns or untranslated regions of protein-coding genes (14). As a result, editing sRNA sequences using the CRISPR system can inadvertently affect protein sequences and production of the proteins encoded by their host loci, resulting in genome-edited strains that truly are double mutants lacking both the sRNAs and protein(s) made from the same primary transcripts. Here, we reported the disruption of the inverted repeat forming the *MIR910* precursor, which is embedded in the intron of Cre14.g629200 annotated as *FLAGELLAR-ASSOCIATED PROTEIN 98* (*FAP98*). Our reverse transcription-quantitative PCR (RT-qPCR) analysis using a primer flanking the intron showed similar *FAP98* mRNA levels in *mir910-1* as in the wild type (*SI Appendix*, Fig. S12), indicating that the effect of the insertion of the short donor sequence on splicing was negligible. The abundance of Cre13.g605300 transcript, containing the *CRS49* precursor, was slightly lower in *crs49-1*, suggesting that the insertion of donor DNA may have affected mRNA stability. It is also conceivable that the stem-loop structure spanning from the open reading frame to the 3′ untranslated region is efficiently processed by the microprocessor prior to translation initiation, limiting the effect on the abundance of protein encoded by Cre13.g605300. These experiments show that CRISPR/Cas9-driven genome editing is an effective method for studying the action and physiological function of individual sRNAs embedded in introns.

In *Chlamydomonas*, LHCSR-mediated qE reduces quantum yield of photosystem II by dissipating the light energy captured by the antennae, which is a light-induced event to protect the photosynthetic apparatus from excessive light (20, 41). The induction of qE should be tightly controlled, so as not to lower photosynthetic efficiency when the light intensity is not high enough to damage the photosynthetic apparatus, such as on cloudy days with light conditions like those affecting the induction of *LHCSR3* expression in this study. We therefore hypothesize that sRNAs function to prevent such inefficient photosynthesis under low light, but we observed no marked difference in growth rate between the wild-type and the miRNA-defective mutants in this study, so observations under complex stress conditions may be necessary to manifest the effects of reduced energy production efficiency due to excess qE.

Red-light irradiation also subtly enhanced *LHCSR3* expression in *mir910-1 crs49-1* (Fig. 3), although this effect was much more modest compared to that resulting from blue light. We cannot exclude the possibility that this slight increase in expression is a result of a synergistic effect between the response to red light and a small amount of blue light (400 to 500 nm, ~1 μmol/m^2/s) in the light emitted by the red light-emitting diode (LED) used in this study. Another possible explanation entails the involvement of miR910 and/or crs49 in the regulation of the red light–dependent induction of *LHCSR3* expression. To explore this possibility, a detailed study of mutants in each photoreceptor in combination with miRNA-defective mutants and *mir910-1 crs49-1* would be required.

In addition to photoprotection, other biological responses in *Chlamydomonas*, including eyespot formation and the controlling sexual life cycle, are regulated by PHOT signals (42, 43), and it is likely that sRNA-mediated *PHOT* regulation also affects them. In land plants, different wavelengths of light provide signals that regulate *MIRNA* expression, miRNA maturation, and accumulation; furthermore, light-dependent photomorphogenesis, circadian clock function, and photoperiod-dependent flowering are controlled by miRNAs (44). Like land plants, *Chlamydomonas* has several photoreceptors (45) that perceive light signals and initiate light-responsive biological phenomena, but the expression and roles of light-dependent sRNAs have not been well studied. It is expected that new roles for sRNAs will be discovered through comprehensive analyses of the light-induced regulation of biological phenomena, changes in gene expression, and sRNA abundance.

Materials and Methods

Algal Strains. Chlamydomonas (*Chlamydomonas reinhardtii*) strain CC-124 was used as the wild type. Gluc(1×) is a transgenic strain expressing a *Gaussia* luciferase reporter gene in the CC-124 background (46). The mutant strains *dus16-1* and *ago3-1* were generated by random insertional mutagenesis in the Gluc(1×) background; their complemented strains were previously described (10, 11). Specific details are provided in *SI Appendix, Materials and Methods*.

Generation of Genome-Edited Mutants and Their Complementation Strains. CRISPR/Cas9-mediated genome editing and expression of artificial miRNAs were performed as previously described (47, 48). Specific details are provided in *SI Appendix, Materials and Methods*.

Culture Conditions. Cells were cultured in Tris-acetate phosphate medium (49) or Sueoka's high-salt medium (50) and used for *LHCSR3* induction. Specific details are provided in *SI Appendix, Materials and Methods*.

psRNAtarget Analysis. Sequences 20 to 23 nucleotides in length with an average of more than 20 counts per million reads obtained from two independent sRNA libraries of Gluc(1×) were selected and used for psRNAtarget analysis. Specific details are provided in *SI Appendix, Materials and Methods*.

RNA Isolation. Total RNA was isolated from cultures with TRI reagent (Molecular Research Center), following the manufacturer's instructions. The concentration was measured using a NanoDrop ND-1000 (Thermo Fisher Scientific).

RT-qPCR Analysis. For first-strand cDNA synthesis, 300 ng total RNA was used as a template with a ReverTra Ace cDNA Synthesis Kit (Toyobo). RT was performed using KOD Fx Neo DNA polymerase, while qPCR was performed using KOD SYBR qPCR Mix (Toyobo) on a StepOne Real-Time PCR System (Thermo Fisher Scientific). The primers are described in *SI Appendix, Materials and Methods*.

sRNA Blotting. sRNAs were detected as previously described (11). Specific details are provided in *SI Appendix, Materials and Methods*.

5'-RACE. This analysis was carried out as previously described (46) and is detailed in *SI Appendix, Materials and Methods*.

Immunoblotting. Protein samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane for chemiluminescence detection. Specific details are provided in *SI Appendix, Materials and Methods*.

NPQ Analysis. Chlorophyll fluorescence-based NPQ measurement was performed. Maximum yields (Fm) were measured under dark conditions (after weak far-red [<5 μmol/m^2/s] treatment for 30 min) using a FluoCam (Photon System Instruments, Czech Republic). The maximum and steady-state fluorescence yields under light (Fm′ and F, respectively) were measured after actinic irradiation at 750 μmol/m^2/s for 30 s. NPQ was estimated using the equation NPQ = (Fm − Fm′)/Fm′

Accession Numbers. Sequence data for the genes discussed in this article can be found in Phytozome v13 (https://phytozome-next.jgi.doe.gov/info/Creinhardtii_v5_6) under the following IDs: Cre14.g629200 (encoding the primary transcript of miR910), Cre13.g605300 (encoding the primary transcript of crs49), Cre03.g199000 (*PHOT*), Cre08.g367400 (*LHCSR3.2*), Cre06.g278222 (*CBLP*), Cre08.g378550 (*NUOP4*), and Cre17.g713200 (*OMT2*).

The sRNA-Seq raw data have been deposited in the DDBJ sequence read archive (DRA) under the accession number DRA003930 (10, 11).

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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