A Systematic Survey of the Light/Dark-dependent Protein Degradation Events in a Model Cyanobacterium

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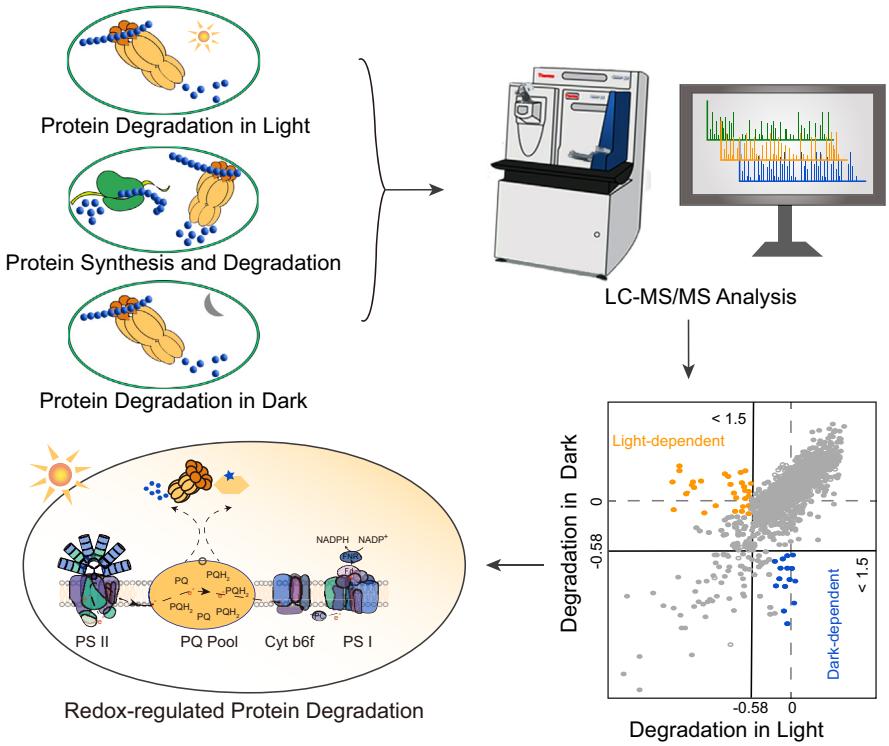
In Brief

Large-scale identification of the light/dark-regulated protein degradation events in the model cyanobacterium *Synechocystis* sp. PCC 6803 was conducted using quantitative proteomics. Proteins with strong degradation in light, dark, or both conditions were identified. The light-regulated degradations of multiple proteins were sensitive to photosynthetic electron transport inhibitors (DCMU and DBMIB), indicative of the redox regulation by the plastoquinone (PQ) pool in the photosynthetic electron transport chain.

Graphical Abstract



Highlights

- Light-/dark-regulated protein degradation events in a model Cyanobacterium were identified.
- Seventy-nine proteins displayed light-regulated degradation.
- Thirty-one proteins displayed dark-regulated degradation.
- Multiple light-regulated protein degradation events were regulated by the redox state of the plastoquinone pool.

2021, Mol Cell Proteomics *20*, 100162

https://doi.org/10.1016/j.mcpro.2021.100162

A Systematic Survey of the Light/Dark-dependent Protein Degradation Events in a Model Cyanobacterium

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Light is essential for photosynthetic organisms and is involved in the regulation of protein synthesis and degradation. The significance of light-regulated protein degradation is exemplified by the well-established light-induced degradation and repair of the photosystem II reaction center D1 protein in higher plants and cyanobacteria. However, systematic studies of light-regulated protein degradation events in photosynthetic organisms are lacking. Thus, we conducted a large-scale survey of protein degradation under light or dark conditions in the model cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter referred to as *Synechocystis*) using the isobaric labeling-based quantitative proteomics technique. The results revealed that 79 proteins showed light-regulated degradation, including proteins involved in photosystem II structure or function, quinone binding, and NADH dehydrogenase. Among these, 25 proteins were strongly dependent on light for degradation. Moreover, the light-dependent degradation of several proteins was sensitive to photosynthetic electron transport inhibitors (DCMU and DBMIB), suggesting that they are influenced by the redox state of the plastoquinone (PQ) pool. Together, our study comprehensively cataloged light-regulated protein degradation events, and the results serve as an important resource for future studies aimed at understanding light-regulated processes and protein quality control mechanisms in cyanobacteria.

Light is essential for photosynthetic organisms by providing energy for photosynthesis and is an important environmental signal regulating photomorphogenesis, which occurs throughout the entire life of higher plants, from germination to seeding development. Light can regulate cellular processes by modulating protein expression at the transcriptional or post-transcriptional level (1, 2). Protein degradation, a crucial process controlling protein abundance and quality, is also regulated by light. It has been reported that the degradation of a number of key regulatory proteins in higher plants is light-induced (3).

One of the important and well-studied light-dependent protein degradation events conserved in both higher plants and photosynthetic cyanobacteria is the light-dependent proteolysis of protein D1 (4–6). D1 is part of the photosystem II (PS II) reaction center and undergoes constant light-induced damage–degradation–repair cycles, which are essential to maintain the activities of PS II and ultimately the optimal operation of photosynthesis (4–6). The light-induced damage–degradation–repair cycle of D1 protein has attracted considerable attention in the last few decades. Proteases involved in degradation of damaged D1 have been identified in higher plants as well as in cyanobacteria (7–9). The PS II reaction center protein D2 also shows light-induced degradation (10, 11), probably due to oxidation of specific amino acid residues by ROS (12). The chloroplast stromal Deg7 protease was reported to be involved in the cleavage of damaged D2 protein (13), but the protease involved in the degradation of D2 in cyanobacteria remains elusive. In addition to the PS II proteins, the blue light photoreceptor family protein CPH1 in *Chlamydomonas reinhardtii* was reported to undergo light-dependent proteolysis, and the process could be affected by a posttranslational modification in its C-terminal region (14). In addition to these proteins, limited information is available regarding the identity and mechanism of light-regulated protein degradation events in photosynthetic organisms. This largely hinders studies seeking to understand how photosynthetic organisms better utilize light while keeping damage to the minimum. Therefore, it is necessary to systematically identify light-regulated protein degradation events and to

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https://doi.org/10.1016/j.mcpro.2021.100162

study their functional significance and the underlying mechanisms.

Here, we used the unicellular photosynthetic model cyanobacterium *Synechocystis* to systematically study light-regulated protein degradation events. *Synechocystis* and other cyanobacteria highly resemble the chloroplasts of higher plants with many important and highly conserved processes, including light-induced damage–degradation–repair of D1 protein (15). *Synechocystis* is the cyanobacterium with the first fully sequenced and probably the best annotated genome (16) and is naturally transformable and thereby amenable to genetic manipulations such as site-directed mutagenesis (17, 18). Such advantages would facilitate large-scale identification of light-regulated protein degradation events and follow-up study of their functional significance. To this end, we treated wild-type (WT) *Synechocystis* cells with a protein synthesis inhibitor and systematically measured the degradation-driven protein abundance changes in light or dark using the tandem mass tag (TMT) labeling-based quantitative proteomics approach. A number of functionally diverse proteins were identified as regulated by light for degradation in addition to D1, D2, and Cph1, and another set of proteins was identified as regulated by dark for degradation. These results provide important information regarding the light/dark-regulated protein quality control mechanism that is critical for maintaining intracellular homeostasis for photosynthetic organisms under constant day/night alternation.

EXPERIMENTAL PROCEDURES

Antibodies

All primary antibodies against *Synechocystis* proteins were provided by Agrisera, except anti-Cph1, which was purchased from PhytoAB. The anti-GFP polyclonal rabbit antibody was purchased from Abcam, and the anti-HA monoclonal mouse antibody was purchased from MBL.

Cell Culture

WT *Synechocystis* (the glucose-tolerant strain) was grown in liquid BG11 medium supplemented with 5 mM glucose at 30 °C to exponential phase ($OD_{730\ nm}$ ~ 1.0) and harvested. The cells were washed and resuspended in fresh liquid BG11 medium at an $OD_{730\ nm}$~0.2 and cultured photoautotrophically to an $OD_{730\ nm}$ ~0.8 with a photosynthetic photon flux density of 50 μmol m^{-2} s^{-1}. The cells were then treated with lincomycin (Sigma Aldrich) at the needed concentrations and incubated in light or in dark for 24 h. Untreated cells in light were used as the control.

Generation of Knock-in Strains Expressing GFP/HA-tagged Proteins

The knock-in strains of *Synechocystis* expressing GFP/HA-tagged target proteins were generated as described previously (19). Briefly, the genomic DNA fragments upstream and downstream from the stop codon of the target genes were cloned by PCR using the primers listed in supplemental Table S1 (KI-F and KI-R) and ligated to the *pEASY*-Blunt Simple vector (Transgen). A fragment containing GFP or HA tag and a kanamycin resistance cassette was inserted into the target gene fragment before the stop codon using the *pEASY*-Uni Seamless Cloning and Assembly Kit (Transgen). The final constructs were used to transform WT *Synechocystis*, and the knock-in strains were selected on solid BG11 plates containing kanamycin. The fully segregated strains were confirmed by PCR as previously described (20).

Protein Preparation

Synechocystis cells were lysed in a buffer containing 0.4 M sucrose, 50 mM MOPS, 10 mM NaCl, 5 mM EDTA (pH 7.0), and 0.5 mM PMSF with a bead beater, and the insoluble debris was removed by centrifugation for 30 min at 5000*g* at 4 °C. The lysates were precipitated with 10% trichloroacetic acid (TCA) in ice-cold acetone at –20 °C and washed with ice-cold acetone to remove pigments, lipids, and residual TCA. The precipitated proteins were air-dried before resolubilization with 4% sodium dodecyl sulfate (SDS) in 0.1 M Tris-HCl, pH 7.6. The protein concentrations were measured using a BCA protein assay kit (Thermo Fisher Scientific).

Protein Digestion and TMT Labeling

Protein digestion and TMT labeling were performed in the same way as described previously (21). Briefly, the proteins were digested with sequencing grade trypsin (Promega) using the filter-aided sample preparation (FASP) method (22). The resultant tryptic peptides were then labeled with 6-plex TMT labeling reagents (Thermo Fisher Scientific). The labeled peptides were mixed together accordingly and lyophilized with a Speed-Vac concentrator.

Peptide Prefractionation and Desalting

The TMT-labeled peptide mixture was separated through reversed-phase (RP)-high-performance liquid chromatography (HPLC) on a Waters e2695 HPLC separation system. Separation was performed on a Gemini-NX 5u C18 column (250 mm × 3.0 mm, 110 Å) (Phenomenex). LC separation was performed as previously reported with a 97 min basic gradient with a flow rate of 0.4 ml/min (23). The separated peptides were combined into 15 fractions and frozen at –20 °C after lyophilization. The dried peptides were resolubilized with 0.5% acetic acid and desalted using C18 StageTips (24). Desalted peptides were dried with a Speed-Vac concentrator, stored at –20 °C, and resuspended in 0.1% formic acid (FA) immediately before LC–MS/MS.

Liquid Chromatography (LC)-Tandem Mass Spectrometry (MS/MS) Analysis

An LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) coupled with an online nanoflow UHPLC system (Thermo Fisher Scientific Easy-nLC 1000) was used for the LC–MS/MS analysis, which was operated in a data-dependent mode. The peptides (2 μl) were separated with a 25-cm-length 75-μm-inner diameter capillary analytic column packed with C18 particles of 5 μm diameter (SunChrom). The LC gradient was composed of 3% to 8% buffer B (buffer B contained 100% ACN and 0.1% FA, whereas buffer A contained 0.1% FA) for 10 min, 8% to 20% buffer B for 60 min, 20% to 30% buffer B for 8 min, 30% to 100% buffer B for 2 min, and 100% buffer B for 10 min. The flow rate was set at 300 nl/min. The source voltage was set at 2.5 KV, and the current was set at 100 μA. MS measurement was performed in positive ion mode. The precursors were measured by survey scans in the Orbitrap with a mass range of 300 to 1800 m/z at a resolution of 120,000 at m/z 400. The 15 most abundant precursor ions (top 15) from each survey scan were selected and fragmented by high-energy collisional dissociation (HCD) for MS/MS analysis. The duration of dynamic exclusion was set to 30 s to prevent repeat identification of peptide ions within the time duration.

Database Search

The raw MS files were searched against the *Synechocystis* proteome sequence database appended with 248 common contaminations using the software MaxQuant (version 1.5.4.1) (25). The database containing 3672 entries was downloaded from CyanoBase (ftp://ftp.kazusa.or.jp/pub/CyanoBase/*Synechocystis*, released on 5/11/2009). The type of LC–MS run was set as reporter ion MS2, and TMT 6-plex was chosen as the isobaric labels. Trypsin/P was set as the protease for protein digestion, and up to two missed cleavages were allowed. The minimum parent ion interference (PIF) was set to 0.75. N-terminal acetylation and methionine oxidation were chosen as the variable modifications, and cysteine carbamidomethylation was chosen as the fixed modification. For precursor ions, the mass tolerance was specified at 20 PPM for the first search and 4.5 PPM for the main search. For fragment ions, the mass tolerance was set to 20 PPM. The 1% false discovery rate (FDR) was set at both the peptide and protein levels. The minimum scores for unmodified peptides and modified peptides were set to 15 and 40, respectively. The match between runs was enabled. All other parameters were set to default values of MaxQuant. Proteins with shared identified peptides were combined and output as a protein group.

Experimental Design and Statistical Rationale

Two TMT labeling-based quantitative experiments were performed to analyze the samples from lincomycin-treated cells in the light and dark. The untreated samples were used as the common control for both experiments, thereby allowing direct comparison of the quantitative results between the two experiments. In each experiment, three biological replicates of the treated samples and of the control were included; therefore, each sample can be labeled by a distinct TMT reagent of the 6-plex TMT. After searching the database, the reporter ion intensities in each TMT channel were normalized based on the assumption that the total reporter ion intensity of each TMT channel should be equal. The medium normalized reporter ion intensities were used to calculate the TMT ratio for each protein between the treated sample and the control.

The software Perseus (version 1.5.4.1) was used for all bioinformatic and statistical analyses (26). Enrichment analysis was performed with Fisher's exact test, and $p < 0.05$ and an enrichment factor >1.5 were used to determine the significance of enrichment.

Chlorophyll (Chl) Fluorescence Measurement

Chl fluorescence was measured using a Fluorometer FluorCam 800 MF (Photon System Instruments, Brno, Czech Republic) as described previously (27). The concentration of the cells was adjusted to $OD_{730\ nm}$~10, and the cells were dark-adapted for 15 min prior to measurement. A 0.8 s flash of saturating light was given to determine Fm. The sensitivity was adjusted to 20%, and the supper was adjusted to 60% for the measurement.

RESULTS

Optimization of Experimental Conditions to Inhibit Protein Translation in Synechocystis *with Lincomycin*

To systematically investigate the protein degradation events and their light dependence in *Synechocystis*, protein synthesis was completely inhibited by lincomycin, an inhibitor widely used to inhibit protein translation in plant chloroplasts and cyanobacteria (28, 29). Upon inhibition of translation, the extent of protein degradation over a specified time duration can be quantified at a proteome scale by measuring the decrease in protein abundance using a quantitative proteomics technique.

Different concentrations of lincomycin have been previously used, ranging from 100 μg/ml to 400 μg/ml, to inhibit translation in *Synechocystis* (29, 30). The optimal working concentration of lincomycin for the present study was determined using two criteria. First, the concentration must be sufficiently high to stop cell proliferation after 24 h of treatment, which is indicative of complete inhibition of protein translation. Second, the cells must be viable after removal of the inhibitor from the culture. This would largely exclude false-positive results from potential cell-death-induced protein degradation. Growth experiments and viability assays were performed to determine the optimal concentration of lincomycin (supplemental Fig. S1). The cells were cultured with a starting concentration at $OD_{730\ nm}$~0.05 or 0.5 in the presence of different concentrations of lincomycin. The growth curves showed that 100 μg/ml lincomycin was sufficient to inhibit the growth of *Synechocystis* after 24 h of treatment, regardless of the starting concentration of the cell culture (supplemental Fig. S1, *A* and *B*).

To test the viability of the cells treated with 100 μg/ml lincomycin for 24 h, the cells were pelleted and washed with fresh culture medium one or two times to remove lincomycin. The cells were then inoculated on solid BG-11 medium and allowed to grow in light. The results show that the viability of lincomycin-treated cells was slightly reduced compared with that of nontreated cells if the cells were washed only once with fresh medium, probably due to the presence of residual lincomycin. Indeed, washing the cells two times with fresh medium nearly completely recovered the viability of the cells (supplemental Fig. S1*C*). Together, the results suggest that treatment with 100 μg/ml lincomycin is sufficient to inhibit protein synthesis while maintaining the viability of the cells.

To ensure that such treatment is sufficiently sensitive to display light-dependent protein degradation, the marker protein D1, a well-known PS II subunit that undergoes a constant damage–degradation–repair cycle in light, was detected in lincomycin-treated or untreated cells in light or in dark by Western blotting (supplemental Fig. S1*D*). As expected, the level of D1 protein in lincomycin-treated cells decreased to a nearly undetectable level compared with that in nontreated cells, whereas in the dark, the levels of D1 protein were nearly equal in both lincomycin-treated and untreated cells. As controls, undetectable degradation was observed for proteins such as HtrA (a serine protease) and AtpB (ATP synthase subunit beta) under the same conditions (supplemental Fig. S1*D*).

High Coverage Identification of the Synechocystis *Proteome*

The strategy for systematic identification of protein degradation events is illustrated in Figure 1. The cells were treated with 100 μg/ml lincomycin for 24 h in the light or dark. The

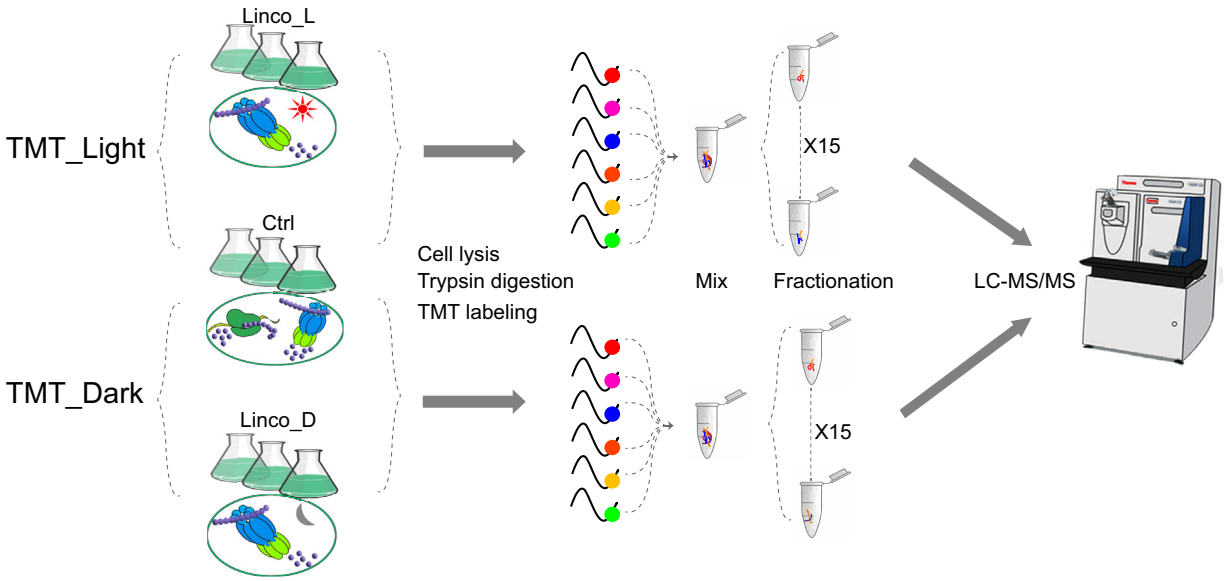

FIG. 1. **Schematic representation of the workflow for quantitative proteomic identification of light/dark regulated protein degradation events in *Synechocystis*.**

untreated cells were used as the control. Three biological replicates were included for each treatment. The protein abundances of lincomycin-treated cells, either in light or in dark, were compared with those of the control cells using a 6-plex TMT-based quantitative proteomic approach. Two independent LC-MS experiments (TMT_Light and TMT_Dark) were performed to measure the protein abundance changes in cells treated with lincomycin under light and dark conditions.

The raw MS files were searched against the *Synechocystis* proteome database using MaxQuant (version 1.5.4.1) (25). In total, 2391 and 2361 proteins were identified with a 1% FDR in the TMT_Light and TMT_Dark experiments, respectively. The identified proteins cover ~65% of the predicted *Synechocystis* proteome (Fig. 2*A*, supplemental Tables S2 and S3) (31). The proteins identified with a single peptide were included, and their MS/MS spectra were manually validated (supplemental Figs. S2 and S3). In addition, 448 transmembrane domain (TM)-containing proteins, as predicted by the software TMHHM 2.0, were identified, which covered more than 50% of the total predicted TM-containing proteins in the *Synechocystis* proteome (Fig. 2*B*). Such high-coverage identification of membrane proteins is particularly important for discovering novel light-regulated protein degradation events, considering that the known proteins with light-regulated degradation, such as D1 and D2, are both membrane proteins. Functional grouping of the identified proteins according to the CyanoBase annotation revealed that identification rates for the majority of groups were higher than 60%, except for two categories, unknown and other categories, which might contain many low-abundance or undetectable proteins (Fig. 2*C*). The high coverage allows identification of novel protein degradation events without a strong bias toward a particular type of function.

Quantitative Determination of Protein Degradation

In the TMT_Light and TMT_Dark experiments, 2167 and 2139 identified proteins contained quantitative TMT information, respectively (supplemental Tables S2 and S3). The reproducibility of the quantitation was evaluated by the pairwise comparison of the normalized reporter ion intensities among the triplicate samples (supplemental Fig. S4). In all comparisons, high correlation coefficients were obtained with a minimum R^2 value equal to 0.979, indicative of high reproducibility of the quantitation.

To filter for the significantly changed proteins, Student's *t* test was performed with $p < 0.05$ as the threshold. In total, 772 and 886 proteins were included, as quantified with high confidence in TMT_Light and TMT_Dark, respectively. To further determine a threshold for the TMT ratios (lincomycin-treated *versus* control), pairwise calculations of the ratios among the triplicate samples were performed (supplemental Fig. S5). In all cases, more than 97% of all quantified proteins showed a less than 1.5-fold change in abundance between any two biological replicates. The result suggests that a fold change of 1.5 is a reasonable threshold for quantitation with an estimated 3% FDR. By applying both Student's *t* test $p < 0.05$ and a fold change of 1.5, 180 proteins and 122 proteins remained with a significant decrease in abundance, *i.e.*, significant degradation, in light and in dark, respectively, and these included 71 proteins overlapping in both conditions (Fig. 3*A*). In line with previous reports (8, 9), the degradation of D1 protein depends on light (Fig. 3*B*). The quantitation was confirmed by manual inspection of the MS/MS spectra of the TMT-labeled peptides from D1 in TMT_Light and TMT_Dark (Fig. 3*C* and supplemental Fig. S6). The light-induced degradation of D1 was further validated by Western blotting (Fig. 3*D*). The previously reported light-induced degradation of D2

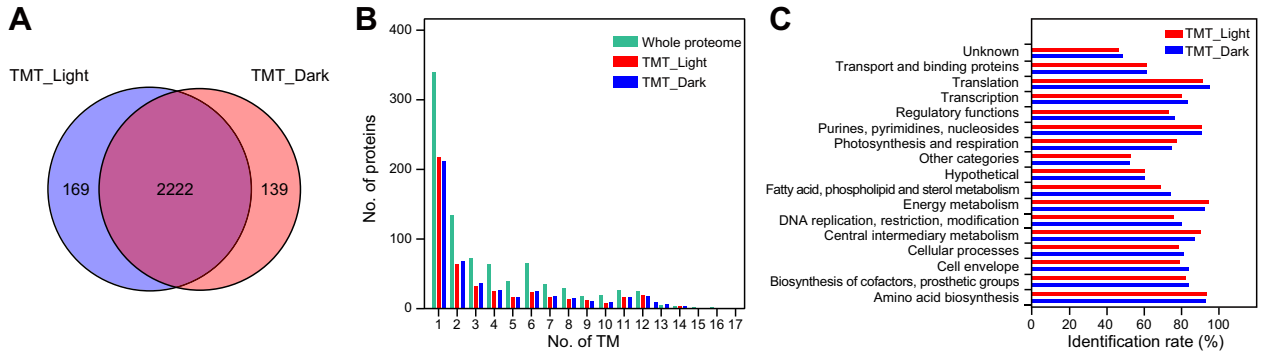

FIG. 2. **Summary of *Synechocystis* proteome identification.** *A*, the Venn diagram shows the numbers of overlapping and unique proteins identified from the lincomycin-treated cells in light and in dark. *B*, the bar graph shows the distribution of the numbers of identified TM-containing proteins in the current study and all TM-containing proteins of the whole *Synechocystis* proteome. The TM is predicted by the software TMHMM (version 2.0). *C*, bar chart representation of the percentage of the identified proteins in each CyanoBase functional category.

was also identified (Fig. 3, *B* and *D*) (10). In contrast, EF-G1 (elongation factor G 1), ClpB1, ClpB2, and NtcA were identified with undetectable degradation, and Rpl1 (50S ribosomal protein L1) was identified with weak degradation in both light and dark (Fig. 3*D*, supplemental Tables S2 and S3). ClpB1 and ClpB2 are two chaperone proteins of the ATP-dependent serine-type Clp protease family (32). NtcA is a transcription factor and acts as a global regulator of nitrogen assimilation and metabolism in cyanobacteria (33).

Enriched Functions Among Proteins With Light-regulated Degradation

Among the 180 proteins with significant degradation in light (Fig. 3*A*), 79 proteins were regulated by light for degradation, including 25 proteins strongly dependent on light for degradation (supplemental Table S4). The degradation of the other 101 proteins was either also significant in the dark or not quantitatively determined in the dark. The enriched functions among the 79 proteins were examined using Fisher's exact test against gene ontology (GO) terms and CyanoBase functional categories (34). As expected, PS II was highly enriched (Fig. 4*A*). In addition to D1 and D2, the two PS II subunits known to exhibit light-induced degradation, the other PS II-related proteins PsbY and Psb27 were also found to exhibit light-regulated degradation (Fig. 3*B*). This result suggests that inhibition of protein synthesis would lead to a significant decrease in PS II levels in the light but not in the dark. Indeed, measurement of Chl fluorescence revealed that QY_max, which represents the maximum quantum efficiency of PS II photochemistry (Fv/Fm), was dramatically decreased in lincomycin-treated cells in light compared with nontreated cells (Fig. 4, *B* and *C* and supplemental Fig. S7). No significant decrease in QY_max was observed for cells treated with lincomycin in the dark (Fig. 4, *B* and *C*). In contrast, the majority of photosystem I (PS I)-related proteins did not display obvious abundance changes under either condition, and no PS I protein displayed light-regulated degradation (Fig. 3*B*). Notably, NADH dehydrogenase activity was also highly enriched among proteins with light-regulated degradation (Fig. 4*A*). In *Arabidopsis thaliana*, rapid turnover of some NAD(P)H dehydrogenase (NDH complex) subunits involved in photoprotection was reported (35); thus, it is not surprising that these proteins can be quickly degraded in light if protein translation is inhibited.

To further confirm the newly identified light-regulated protein degradation events, we generated knock-in mutants with GFP tags fused at the C-terminus for a few proteins with known or unknown functions. These include Cph1, PhaC, PhaE, GifA, Slr0060, and Sll1980. Cph1 is the only protein in the list showing strong degradation in light but without successful identification in the dark due to the stochastic nature of shotgun proteomics. The proteins PhaC and PhaE form the heterodimeric PhaEC synthase, which catalyzes the last step of the synthesis of polyhydroxybutyrate (PHB), a carbon and energy storage compound, under nutrient-limiting conditions (36). GifA, an inactivating factor of glutamine synthetase, was identified as a natively unfolded protein and can be rapidly degraded in cells treated with ammonium (37). Slr0060 and Sll1980 are functionally unknown proteins. Through phenotypic analysis, we found that the growth of Δ*sll1980*, an *sll1980*-knockout strain of *Synechocystis*, was severely inhibited under all tested growth conditions (supplemental Fig. S8). For all *Synechocystis* strains expressing GFP-tagged proteins, growth experiments were performed to confirm that tagging of these proteins does not interfere with the functionality of the tagged proteins in cell growth (supplemental Fig. S9). Probing these tagged proteins by Western blotting revealed that they all significantly decreased in light but hardly decreased in the dark in lincomycin-treated cells (Fig. 4*D*). The light-regulated degradation of WT Cph1 was further confirmed by Western blotting using an anti-Cph1 antibody (supplemental Fig. S10).

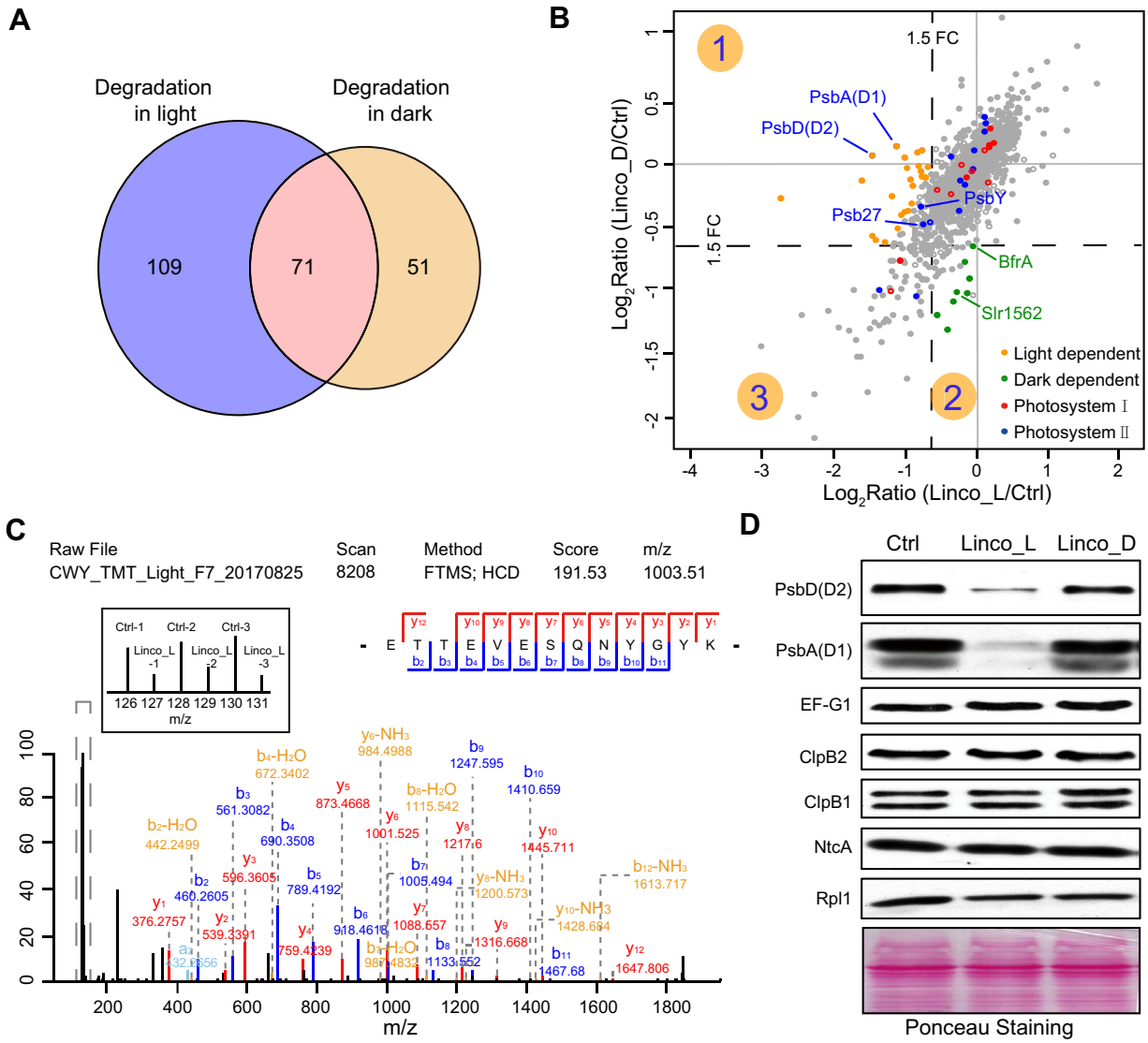

FIG. 3. **Quantitative identification of the *Synechocystis* proteome.** *A*, Venn diagram shows the number of proteins quantified with a significant decrease in abundance in the light (degradation in light) or in the dark (degradation in dark) in lincomycin-treated cells. The overlapping proteins were those with a significant decrease in abundance under both conditions. *B*, the scatter plot displays the comparison of the TMT ratios measured in both conditions. The plot area was separated into four regions by the *dashed lines* representing the threshold of the TMT ratios (1.5-fold change, logarithm-transformed). In region 1, all proteins were significantly decreased in abundance in lincomycin-treated cells in the light but not in the dark. The spots in *orange* indicate the 25 proteins showing strong dependence on light for degradation (TMT ratio (dark/light) > 1.5). Conversely, in region 2, a significant decrease in abundance was observed only in the dark but not in the light. The spots in *green* indicate the eight proteins showing strong dependence on darkness for degradation (TMT ratio (light/dark) > 1.5). In region 3, a significant decrease in abundance was observed for all proteins under both conditions. The X-axis and Y axis: logarithm transformed TMT ratios for light/control (X-axis) and dark/control (Y-axis). *C*, a representative mass spectrum of a peptide from D1 protein identified in the experiment TMT_Light. The bars representing the TMT report ions (boxed by *dashed lines*) are amplified to show the relative abundance of the peptide in each sample. *D*, Western blotting validation of abundance changes for the indicated proteins in control and lincomycin-treated cells. Equal loading is shown by Ponceau staining.

Proteins With Dark-regulated Degradation

In total, 122 proteins were determined to have significant degradation in the dark, including 51 proteins whose abundances were not significantly decreased (31 proteins) or not quantitatively determined (20 proteins) in the light (Fig. 3*A*). Thus, the degradation of the 31 proteins without significant abundance changes in light was deemed dark-regulated (supplemental Table S5), including eight proteins strongly dependent on darkness for degradation (Fig. 3*B*). The functional enrichment analysis revealed that a few GO terms were enriched among the 31 proteins with dark-regulated degradation. These processes include transcription, RNA

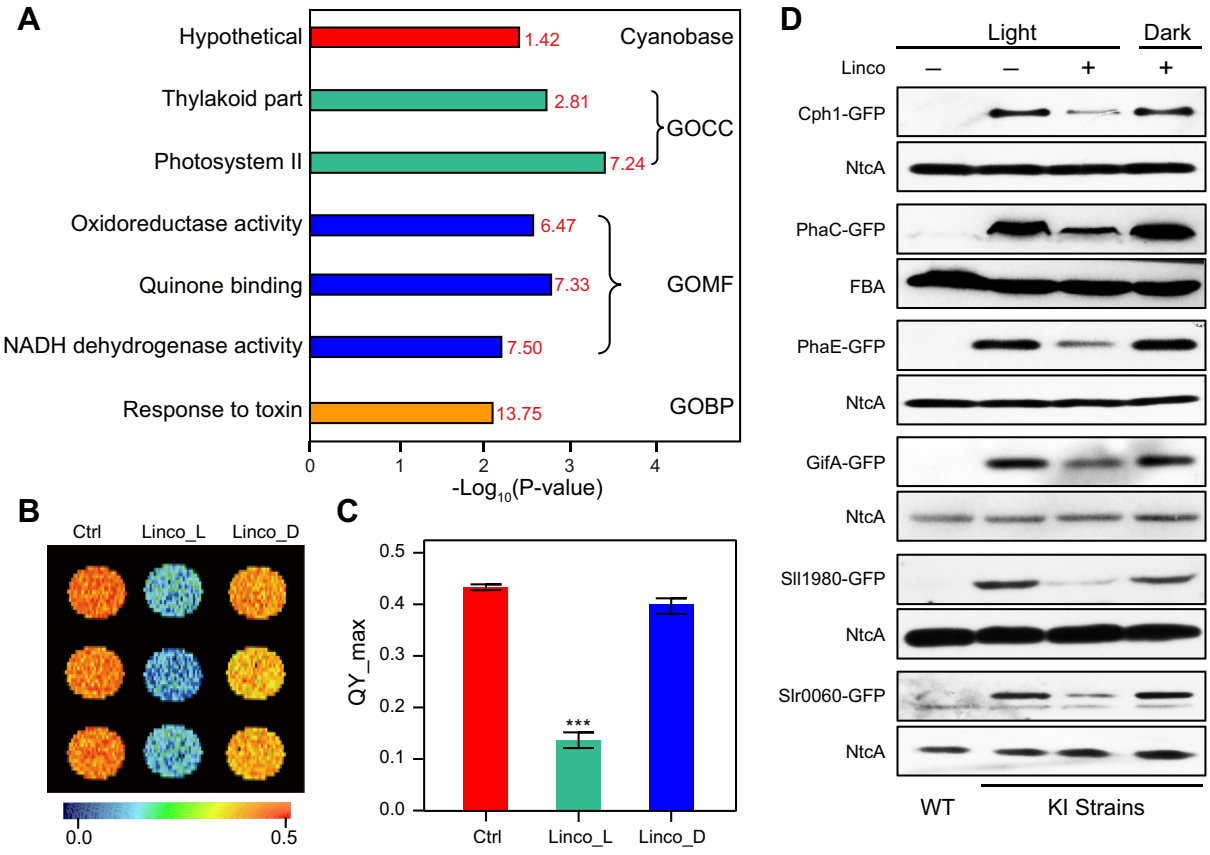

FIG. 4. **Enriched functions of proteins with light-regulated degradation.** *A*, Fisher's exact test for the functional enrichment of proteins with light-regulated degradation. *p* value <0.05 and enrichment factor >1.5 were used as the thresholds. The functional categories annotated by CyanoBase and gene ontology terms (GOCC: Cellular component; GOMF: Molecular function; GOBP: Biological process) were included for enrichment analysis. The enrichment factors are shown in *red*. *B*, Chl fluorescence imaging shows the Fv/Fm (QY_max) of the control and lincomycin-treated cells. The values are shown by the bar graph (*C*). ***p value <0.001. *D*, Western blotting verification of light-regulated degradation of the indicated proteins. NtcA and FBA, which are quite stable in the current experimental conditions, were also probed as loading controls. Representative results from at least three independent experiments are shown. KI, Knock-in.

biosynthetic processes, biological regulation, and regulation of primary metabolic processes (Fig. 5*A*). Slr1562 and BfrA (Sll1341), which are annotated as being involved in biological regulation, displayed dark-dependent degradation (Fig. 3*B*). The redox protein Slr1562 (also known as Grx1 or GrxB) is a glutaredoxin that is important for the adaptation to oxidative stress in *Synechocystis* (38). Together, these findings indicate that in the dark, certain proteins, particularly regulatory proteins, also undergo active degradation and conceivable repair cycles.

Proteins Undergoing Degradation in Both Light and Dark

In total, 71 proteins showed significant degradation both in light and in dark (Fig. 3*A* and supplemental Table S6). Functional enrichment analysis indicated that ribosomal proteins and proteins involved in folate biosynthesis were over-represented among these proteins (Fig. 5*B*). Ribosomal proteins, and probably folate biosynthesis proteins as well, perform housekeeping functions irrespective of light or dark conditions. Significant degradation of these proteins under both conditions suggests that they could be damaged during active translation or folate biosynthesis processes, and the damaged proteins need to be removed and substituted with newly synthesized copies. Interestingly, the proteins encoded by the genes located on the plasmid pSYSX were also over-represented (Fig. 5*B*), suggesting that they also have a higher turnover rate.

Redox-regulated Protein Degradation in Synechocystis

For proteins displaying light-dependent degradation, one of the prominent questions remains how light controls this process. Light-dark transition could change the intracellular redox status (39, 40). In general, the intracellular environment becomes more reduced when cells are under light irradiation because of the production of reducing power equivalents (*e.g.*, NADPH) by the photosynthetic electron transport (PET) chain (39, 40). In the dark, the intracellular environment shifts toward more oxidation due to the consumption of reducing

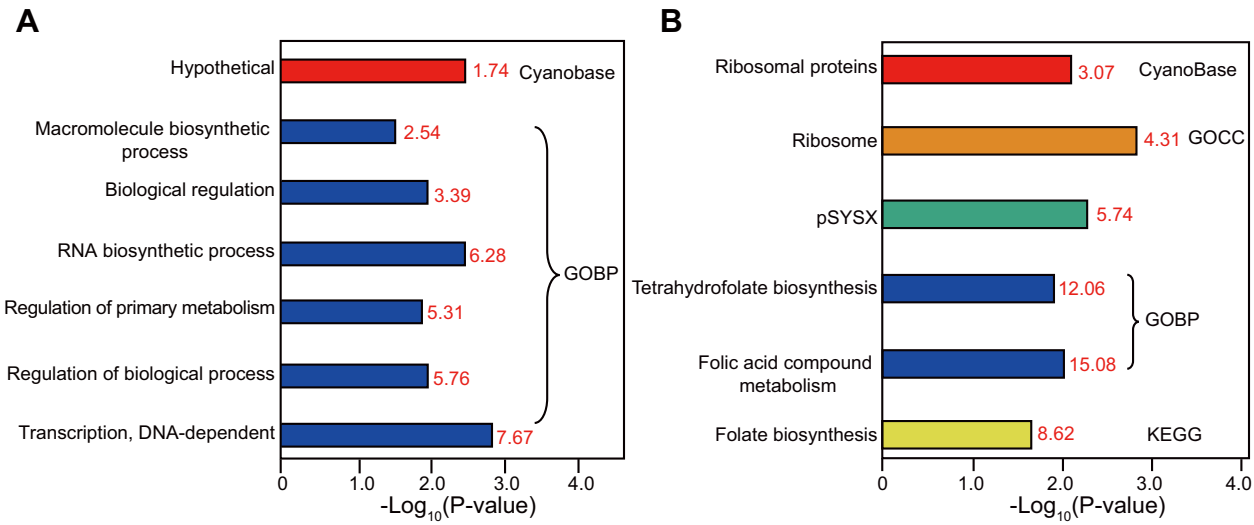

FIG. 5. **Enriched functions of proteins with light-independent degradation.** Fisher's exact test for the functional enrichment of proteins with dark-regulated degradation (*A*) and proteins with significant degradation both in light and in dark (*B*). The test was performed as shown in Figure 4*A*, and the same thresholds were applied.

equivalents by metabolic processes (39, 40). Thus, it is possible that light controls protein degradation by regulating intracellular redox status, at least for a subset of proteins.

To test this hypothesis, we treated lincomycin-treated cells with the oxidant H_2O_2 or the reductant DTT and detected the degradation of PhaE, a protein showing light-dependent degradation (Fig. 4*D* and supplemental Table S4). As expected, treatment with H_2O_2 almost completely inhibited the light-dependent degradation of PhaE, and no remarkable difference at the protein level was observed compared with the control (Fig. 6*A*). Moreover, treatment with methyl viologen (MV), an alternative oxidant sequestering electrons from PS I and oxidizing PET (41), also significantly inhibited the degradation of PhaE in light (Fig. 6*A*). Consistently, DTT, a reducing agent, reactivated dark-inhibited PhaE degradation in lincomycin-treated cells, although the degradation was to a lesser extent than that in light-treated cells (Fig. 6*A*). Together, these results strongly suggest that PhaE degradation is regulated by intracellular redox status and is favored by a reducing status.

To further confirm that redox changes can regulate PhaE degradation, we also probed PhaE degradation in lincomycin-treated cells in the dark in the presence or absence of glucose. In the dark, exogenously supplied glucose can be catabolized through the oxidative pentose phosphate pathway and generate NADPH (39), thereby shifting the intracellular redox status toward reduction. As expected, glucose treatment promoted significant degradation of PhaE, which otherwise showed negligible degradation in the dark (Fig. 6*B*). Notably, glucose treatment promoted PhaE degradation in lincomycin-treated cells to a greater extent in light than in the dark (Fig. 6*B*), probably because the intracellular environment is reduced more in light.

To further investigate whether PhaE degradation is correlated with the redox status of the PQ pool, an electron sink in PET directly responsive to light–dark transition (40), we treated lincomycin-treated cells with DCMU and DBMIB, two widely used electron transport inhibitors that can modulate the redox status of the PQ pool. DCMU blocks electron transport from PS II to the PQ pool and thereby oxidizes the PQ pool, and DBMIB blocks electron transport from the PQ pool to the cytochrome b_6f complex and thereby reduces the PQ pool (40, 42). DCMU almost completely abolished PhaE degradation in lincomycin-treated cells in light (Fig. 6*C*) compared with the results without DCMU treatment (Figs. 4*D* and 6*A*). In line with this, DBMIB strongly induced PhaE degradation in lincomycin-treated cells in the dark (Fig. 6*C*), which is in sharp contrast with the results from the experiments without DBMIB treatment (Figs. 4*D* and 6*A*). Remarkably, the extent of DBMIB-induced degradation was greater in the light than in the dark (Fig. 6*C*), probably because of the greater reduction in the PQ pool in the light. Notably, a high concentration (100 μM) of DBMIB can block electron transfer from Q_A to Q_B of PSII (43). In the current study, the concentration of DBMIB was 10 μM. At this concentration, DBMIB can completely block electron transfer from the PQ pool to the cytochrome b_6f complex with only a negligible effect on electron transfer from Q_A to Q_B of PS II (43) and can significantly inhibit the growth of cells (supplemental Fig. S11). Moreover, 50 μM DBMIB and dark incubation can cause a reduction in the PQ pool, as recently measured by Khorobrykh *et al.* (44).

In addition to PS II, the respiratory electron chain can also supply electrons to the PQ pools by catabolizing glucose (45, 46). Indeed, DCMU-inhibited light-dependent degradation of PhaE was reactivated by exogenously supplied glucose (Fig. 6*D*). Together, these results strongly suggest that the redox

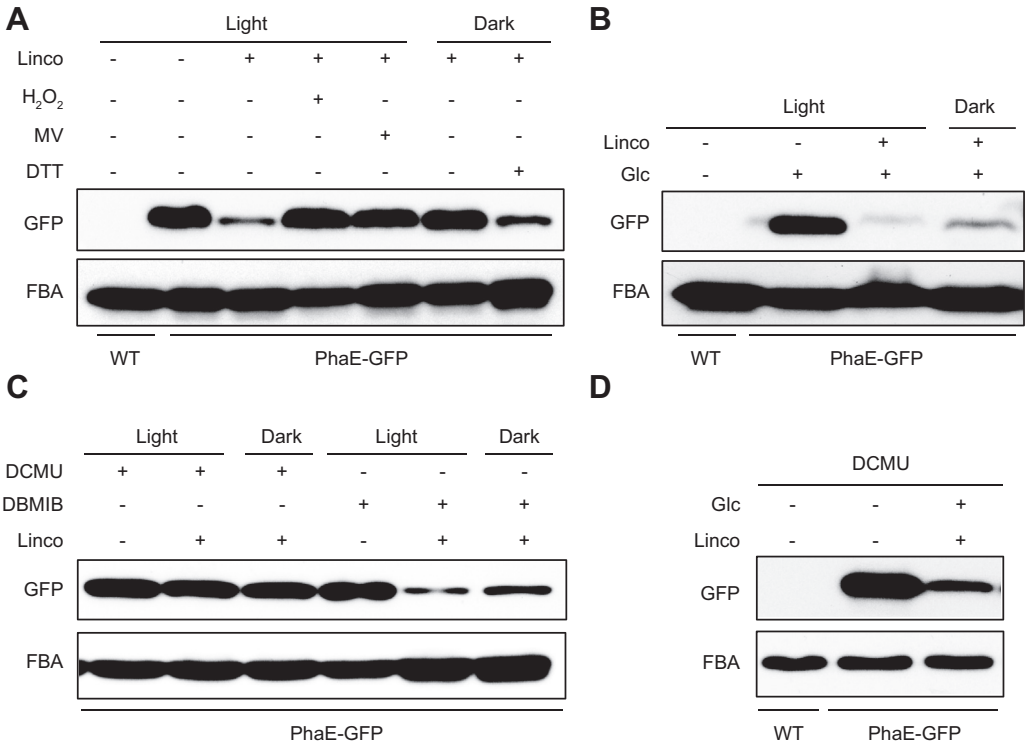

FIG. 6. **Redox regulation of PhaE degradation.** Western blotting detection of PhaE-GFP in lincomycin-treated cells that were also treated with different reagents. *A*, H_2O_2 (1 mM), DTT (20 mM), or MV (2 μM). *B*, glucose (5 mM), (*C*) DCMU (10 μM) or DBMIB (10 μM), (*D*) DCMU (10 μM) and/or glucose (5 mM). The treatments were performed either in light or in dark as indicated. Cells without lincomycin treatment were used as the control.

status of the intracellular environment and the PQ pool play a pivotal role in regulating PhaE degradation.

To determine whether redox-regulated PhaE degradation is a unique case or a more general mechanism for light-regulated protein quality control, we further tested redox regulation of degradation for three other proteins, including Slr0060, PhaC, and ClpX, in a similar way. All three proteins showed light-dependent degradation (Fig. 4*D* and supplemental Table S4). Again, in lincomycin-treated cells, glucose promoted the degradation of all three proteins in the dark (Fig. 7, *A*, *C* and *E*), whereas DCMU blocked their degradation in light (Fig. 7, *B*, *D* and *F*). DBMIB also induced degradation of all three proteins in the dark, although the degradation of PhaC was much weaker than that of the other two proteins (Fig. 7, *B*, *D* and *F*). Together, these data suggest that redox regulation, probably of the PQ pool, is a more general mechanism underlying light-dependent protein degradation (Fig. 8).

DISCUSSION

In the present study, we identified 79, 31, and 71 proteins from lincomycin-treated *Synechocystis* that showed strong degradation in light, dark, or both conditions. We then showed that light-dependent degradation of a subset of proteins is regulated by the redox status of the intracellular environment and the PQ pool (Fig. 8). A more systematic investigation is needed to answer whether such redox regulation is a general mechanism involved in light-induced protein quality control in photosynthetic organisms. Nevertheless, the current results provide numerous important targets to study light-regulated processes in photosynthetic organisms. It is remarkable that only medium light intensity was used in the current study to induce protein degradation for the purpose of probing the light-regulated protein degradation events in a more physiologically relevant condition. High light intensity could also be used if the detection of more dramatic protein degradation events is expected under stressful conditions. Moreover, 24 h instead of 12 h incubation with lincomycin in light or in dark was used in the current study, and the latter is more physiologically relevant as it better mimics day–night cycles. We reasoned that 24 h of incubation could elongate the degradation process and thereby magnify the differences in protein abundances before and after the degradation process and would increase the sensitivity for detecting light- or dark-regulated protein degradation events. Substantial degradation at 12 h was also confirmed by Western blotting for several proteins with significant degradation at 24 h (supplemental Fig. S12).

Including D1 and D2 proteins, 25 proteins with different functions showed light-dependent degradation (Fig. 3*B* and

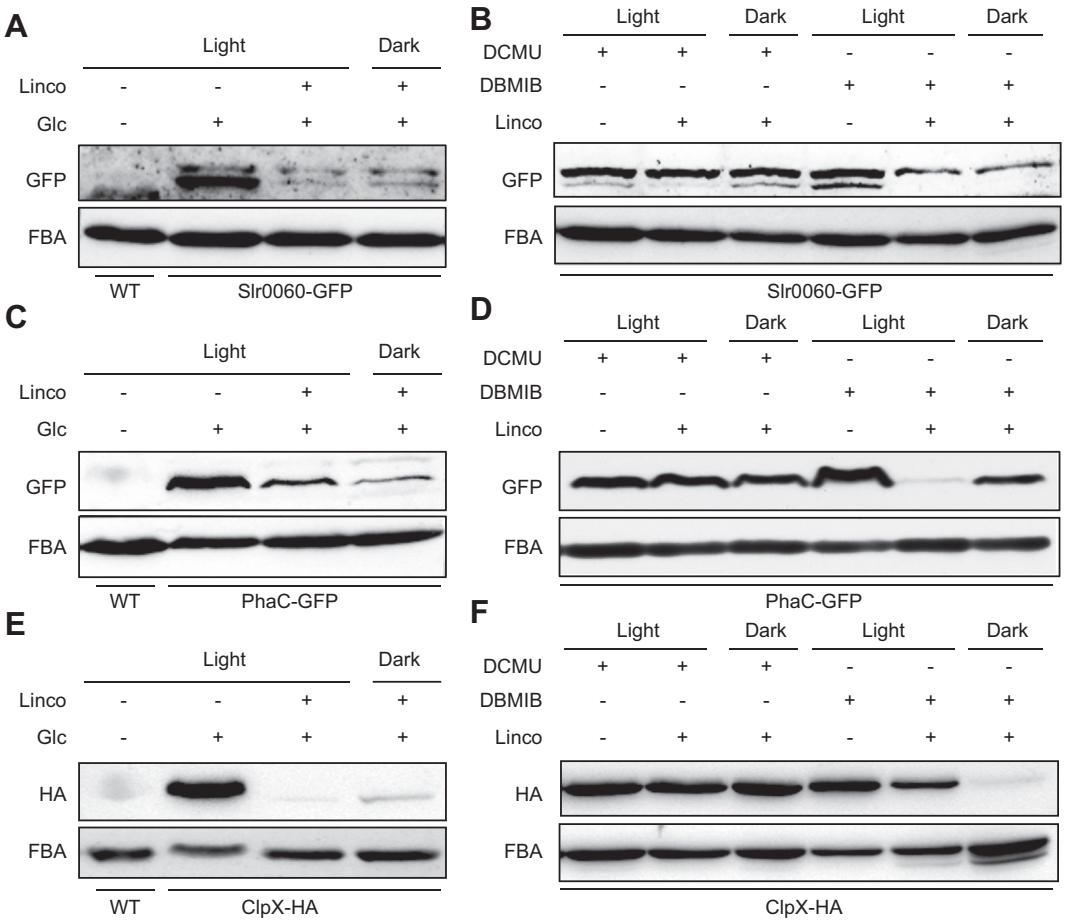

FIG. 7. **Redox-regulated degradation of Slr0060, PhaC, and ClpX.** Western blotting detection of Slr0060 (*A* and *B*), PhaC (*C* and *D*), and ClpX (*E* and *F*) in lincomycin-treated or untreated cells in the presence or absence of glucose (5 mM) (*A*, *C* and *E*) or DCMU or DBMIB (*B*, *D* and *F*). The treatments were performed either in light or in dark as indicated.

supplemental Table S4). One of the important questions remains the physiological significance of such light-dependent protein degradation. The significance of D1 degradation is well known as an integrative step in the light-induced damage–degradation–repair cycle of the PS II complex (4, 28). It is possible that other proteins may share a similar mechanism to maintain optimal functionality in light. Inactivation of the cognate proteases could inhibit the light-dependent degradation events and facilitate addressing their functional significance. Although a follow-up study including the identification of cognate proteases is ongoing, it is beyond the scope of the current study.

In addition to the proteins with known functions, some proteins with unknown functions also displayed light-dependent protein degradation, including Sll1980 and Slr0060 (Fig. 4*D*). Sll1980 is predicted to be a thioredoxin-like protein with unknown function. Thioredoxins act as redox carriers and participate in the light-dependent regulation of enzymes in photosynthetic organisms (47). The light-dependent degradation of Sll1980 might be due to light-induced redox modification on certain amino acid residues. Genetic inactivation of *sll1980* significantly inhibited the trophic growth of *Synechocystis* (supplemental Fig. S8), yet the significance of the light-dependent degradation of Sll1980 *per se* remains to be addressed. The hypothetical protein Slr0060 is encoded by a gene within an operon (*slr0058-slr0061*) involved in polyhydroxybutyrate (PHB) biosynthesis (48). Genetic inactivation of Slr0060 did not cause observable defects in growth and PHB biosynthesis in *Synechocystis* (48). Again, the function of Slr0060 and the significance of its light-dependent degradation remain to be addressed.

In addition to the proteins showing light-dependent degradation, eight proteins showed a strong dependence on the dark for degradation (Fig. 3*B* and supplemental Table S5). Light-dependent protein degradation for a number of proteins was regulated by the redox status of the intracellular environment and the PQ pool, and it is conceivable that dark-dependent protein degradation could also be regulated by the redox status. Shifting the redox of the intracellular environment and the PQ pool toward reduction favors light-dependent protein degradation (Figs. 6 and 7). Similarly, shifting the redox status toward oxidization could favor

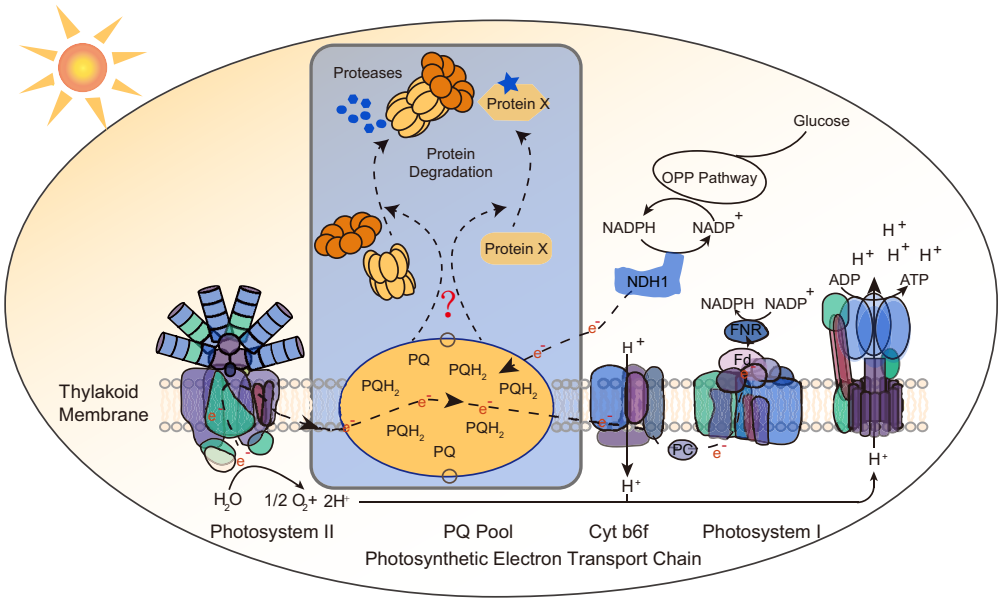

FIG. 8. **A working model for redox-regulated protein degradation in *Synechocystis*.** All electron carriers of PET are depicted, and the mobile PQ pool (reduced PQH_2 and oxidized PQ) is highlighted. The PQ pool could become more reduced through the metabolism of glucose.

dark-dependent protein degradation. Although the redox of the PQ pool can be regulated in feedback through cytochrome *bd*-type oxidase, particularly under high light intensity, to prevent its overreduction (49), the redox difference of the PQ pool between light and dark is significant, as reported by Khorobrykh *et al.* (44). Together, these results could reveal the mechanism of circadian protein quality control by changing the intracellular and PQ pool redox status.

The mechanism underlying the dependence of protein degradation on light/dark is largely unknown except for D1. However, two major facts could be attributed to the differential dependence. The first is the damage (similar to D1) or redox modification that changes the conformation or stability of the target proteins (12). The target proteins become dysfunctional and recruit cognate proteases for degradation. Indeed, many proteins are differentially redox modified when the cells are placed in light or dark (50, 51), including PhaE (50). Identification of the specific redox modification sites on these proteins could help to investigate the functions and mechanisms of light/dark-dependent degradation (50). The second is the differential activation of cognate proteases in light or in the dark, probably through redox modification of the proteases. In line with this, the activity of a number of proteases is regulated by redox status (52, 53).

It is worth noting that in general, the abundances of the majority of proteins remain relatively stable despite significant oscillation of transcription and translation during light/dark cycles (54–56). Slow bulk protein turnover, but not gene-specific protein turnover, was suggested to be the major contributor to maintaining such stability through *in silico* modeling (54).

In conclusion, the current study took an important step toward understanding the protein quality control mechanism for photosynthetic organisms in response to alternating day–night transitions and revealed that intracellular redox status plays an important role in regulating light-dependent protein degradation. The results should serve as an important resource for understanding the light-regulated processes in photosynthetic organisms.

DATA AVAILABILITY

The raw mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE (57) partner repository with the dataset identifier PXD023181.

Supplemental data—This article contains supplemental data.

Acknowledgments—We thank Dr. Yuanya Zhang for the assistance in mass spectrometry and Xiaoxiao Duan and Zhen Xiao for the constructive suggestions in data analysis.

Funding and additional information—The work was supported by a grant from National Natural Science Foundation of China (31670234 to Y. W.) and the grant 2019YFA0802203 from the Ministry of Science and Technology of China.

Author contributions—W. C., W. X., and Yingchun Wang conceptualization; W. C., L. Z., J. D., H. G., X. H., D. L., and Yingchun Wang methodology; W. C., L. Z., J. D., G. W., C. H., and Yan Wang investigation; W. C. and H. G. visualization; W. C. writing–original draft; L. Z., J. D., H. G., X. H., G. W., C. H.,

Yan Wang and Yingchun Wang data curation; X. H. project administration; D. L., W. X., and Yingchun Wang writing–review and editing; Yingchun Wang formal analysis; Yingchun Wang resources.

Conflict of interest—The authors declare no competing interests.

Abbreviations—The abbreviations used are: Chl, chlorophyll; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FASP, filter-aided sample preparation; FDR, false discovery rate; HCD, higher-energy collisional dissociation; LC–MS, liquid chromatography–mass spectrometry; Linco, lincomycin; MOPS, 3-(N-morpholino) propanesulfonic acid; MS, mass spectrometry; PET, photosynthetic electron transport chain; PIF, parent ion interference; PMSF, phenylmethylsulfonyl fluoride; PQ, plastoquinone; RP-HPLC, reversed phase–high-performance liquid chromatography; *Synechocystis*, *Synechocystis* sp. PCC 6803; TMT, tandem mass tag; WT, wild type.

Received March 26, 2021, and in revised from, September 8, 2021
Published, MCPRO Papers in Press, October 13, 2021, https://doi.org/10.1016/j.mcpro.2021.100162

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