Photosystem II Subunit PsbS Is Involved in the Induction of LHCSR Protein-dependent Energy Dissipation in Chlamydomonas reinhardtii

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Non-photochemical quenching of excess excitation energy is an important photoprotective mechanism in photosynthetic organisms. In Arabidopsis thaliana, a high quenching capacity is constitutively present and depends on the PsbS protein. In the green alga Chlamydomonas reinhardtii, non-photochemical quenching becomes activated upon high light acclimation and requires the accumulation of light harvesting complex stress-related (LHCSR) proteins. Expression of the PsbS protein in C. reinhardtii has not been reported yet. Here, we show that PsbS is a light-induced protein in C. reinhardtii, whose accumulation under high light is further controlled by CO₂ availability. PsbS accumulated after several hours of high light illumination at low CO₂. At high CO₂, however, PsbS was only transiently expressed under high light and was degraded after 1 h of high light exposure. PsbS accumulation correlated with an enhanced non-photochemical quenching capacity in high light-acclimated cells grown at low CO₂. However, PsbS could not compensate for the function of LHCSR in an LHCSR-deficient mutant. Knockdown of PsbS accumulation led to reduction of both non-photochemical quenching capacity and LHCSR3 accumulation. Our data suggest that PsbS is essential for the activation of non-photochemical quenching in C. reinhardtii, possibly by promoting conformational changes required for activation of LHCSR3-dependent quenching in the antenna of photosystem II.

Sunlight is the ultimate energy source for photosynthesis. Although efficient light absorption is essential for efficient photosynthesis, the absorption of light energy in excess of its utilization in photosynthesis may lead to the production of reactive oxygen species, which cause cell damage (1, 2). Oxygenic photosynthesis evolved in cyanobacteria and was transferred laterally to eukaryotes through endosymbiosis (3), and both cyanobacteria and photosynthetic eukaryotes have evolved photoprotective mechanisms allowing the dissipation of excess light energy as heat (4). One of the most important and pervasive mechanisms of minimizing photo-oxidative damage by excess light energy is the harmless deactivation of singlet excited chlorophylls in the light harvesting antenna as heat, known as non-photochemical quenching (NPQ). In land plants, NPQ is composed of different components, with the so-called qE-component being the dominant mechanism under most natural conditions (5). The qE mechanism is strictly regulated by changes in the pH of the thylakoid lumen (6) and thus operates on a very short time scale allowing a rapid response of the photosynthetic machinery to rapid changes in light intensities.

Although the function of qE is the same in green algae and plants, the underlying mechanisms differ among green algae and plants (7). In the green alga Chlamydomonas reinhardtii, qE depends on light-induced accumulation of the LHCSR proteins, specifically LHCSR3 (8). LHCSR3 is a pigment binding member of the LHC family that is activated via protonation of acidic amino acid residues upon acidification of the lumen, allowing for the reversible switch from a light harvesting to a dissipative state (9–11). In land plants, the qE mechanism differs between vascular and non-vascular plants. In the moss Physcomitrella patens, qE is independently and additively activated by two types of proteins: LHCSR3 (as in green algae) and PsbS, a non-pigment binding protein from the LHC family (12, 13). Both proteins are activated in parallel by changes in the thylakoid luminal pH, but contrary to C. reinhardtii (14, 15), pH-regulated synthesis of zeaxanthin from violaxanthin also significantly contributes to qE. In particular, zeaxanthin binding to LHCSR enhances the LHCSR-dependent qE capacity (16). PsbS, on the other hand, is supposed to enhance the quenching of excess energy by direct interaction with LHCCI trimer proteins (17). In vascular plants, qE requires pH-regulated activation of PsbS by protonation of two glutamate residues at its two luminal loops (18–20) and is further modulated

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6 The abbreviations used are: NPQ, non-photochemical quenching; HL, high light; HS, high salt; LHC, light-harvesting complex; LHCSR, light-harvesting complex stress-related; LL, low light; PSI, photosystem I; PSII, photosystem II; qE, energy-dependent quenching; TAP, tris-acetate-phosphate; amiRNA, artificial micro RNA; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propan-1,3-diol.
by zeaxanthin synthesis (21). The qE mechanism has been extensively studied in Arabidopsis thaliana, a model organism for the study of energy dissipation mechanisms since the essential role of PsbS in qE activation was described (18). In the current model for qE activation, acidification of the lumen activates PsbS and thus facilitates conformational changes in the LHCCI-PSII supercomplexes, thereby promoting a rearrangement of LHCCI (22–25). Activation of qE in A. thaliana is accompanied by an increased interaction of PsbS with trimeric LHCCI and PSII reaction center proteins (23). It should be noted, however, that under in vitro conditions, qE can be induced even in the absence of PsbS, when the lumen pH is artificially lowered below pH 5.5, indicating that PsbS controls the pH sensitivity of conformational changes required for qE (26).

PsbS homologs have been found in several lineages of green algae (15, 27, 28). In C. reinhardtii, two PsbS-encoding genes (PSBS1 and PSBS2) have been identified (29). The encoded proteins differ only in one amino acid of the chloroplast transit peptide, and they show 48% similarity with A. thaliana PsbS, including the two pH-sensing glutamate residues at the luminal side (15). Transcriptomic analyses have revealed that mRNA levels of the two PsbS-encoding genes in C. reinhardtii (PSBS1 and PSBS2) are up-regulated upon nitrogen starvation (30) and after a shift from dark or low light to higher light intensities (31–33). However, expression and accumulation of the PsbS protein has not been reported so far, and earlier work showed that overexpressed PsbS does not localize to the thylakoid membrane in C. reinhardtii (27). In this work we show that PsbS transiently accumulates in C. reinhardtii during activation of qE upon high light acclimation and that the accumulation of PsbS compared with the photosystem II reaction center proteins but was enhanced in their absence. Knockdown of PsbS expression leads to a pronounced reduction of LHCSR accumulation and qE activation. Our data suggest that PsbS is required for the activation of the qE capacity elicited by the LHCSR protein upon high light acclimation in C. reinhardtii.

Results

Inducible Expression of the PsbS Protein—Based on the predicted PsbS protein sequence (15), we designed an antibody that specifically recognizes PsbS of C. reinhardtii. The antibody binds to the C-terminal sequence of the protein, which is identical for the proteins encoded by the two genes PSBS1 and PSBS2. Its reactivity was confirmed by immunodetection of partially purified recombinant CrPsbS expressed in Escherichia coli (Fig. 1A) and total protein extracts from CrPsbS-expressing E. coli cells (Fig. 1B). The antibody showed no cross-reaction with the A. thaliana PsbS (Fig. 1B).

At the transcript level, PsbS mRNA was shown to increase upon nitrogen starvation under mixotrophic growth (30) and after transferring photoautotrophically grown cells from dark or low light to high light (31–33). To identify specific conditions that trigger expression of the PsbS protein, we designed an experiment combining different light conditions and carbon and nitrogen availabilities. Cells were grown under low light (LL) or high light (HL) under either mixotrophic (TAP medium with acetate as a carbon source) or photoautotrophic (high salt minimal medium (HS)) conditions and in the presence or absence of a nitrogen source. In wild-type (WT) cells, PsbS was expressed in HL-acclimated cells grown under photoautotrophic conditions, and PsbS accumulation was reduced upon nitrogen starvation (Fig. 2A). No PsbS protein was detected in LL-grown cells or in mixotrophically grown cells (Fig. 2A). It should be noted that PsbS protein could only be detected by immunoblot analysis when the amount of total protein loaded on the gel was increased to 40 μg instead of 5 μg for other proteins (Fig. 2), which might indicate a substoichiometric accumulation of PsbS compared with the photosystem II (PSII) reaction center core subunit D1 and LHCSR.

In the npq4lhcsr1 double mutant, which lacks functional LHCSR3 and LHCSR1 (11), PsbS was more strongly expressed compared with the WT and was not degraded upon nitrogen starvation (Fig. 2B). Consequently, the light-induced expression and accumulation of PsbS did not depend on the accumulation of LHCSR proteins but was enhanced in their absence. LHCSR amounts were higher under photoautotrophic conditions (HS medium) in both LL- and HL-grown cells compared with cells grown under mixotrophic conditions (TAP medium) in HL.

PsbS Expression during High Light Acclimation at Different CO2 Availabilities—The responses of C. reinhardtii to HL are commonly studied by transferring LL-grown cells to HL, thus simulating long term acclimation to HL upon increases in light intensities. Monitoring the expression of PsbS (and other proteins) under constant HL growth may thus not be an appropriate system to study the function of this protein, because PSBS mRNA is only transiently up-regulated upon transition from low to high light intensities (31, 32). Additionally, the energy quenching capacity in C. reinhardtii also depends on CO2 availability (34–36). Thus, if the function of PsbS is related to the activation of qE under HL, CO2-dependent changes in its expression are expected. To address these questions, WT and
**PsbS Function in C. reinhardtii**

**FIGURE 2. PsbS expression under different light, carbon, and nitrogen conditions.** Cells were grown under LL (30 μmol photons m⁻² s⁻¹) or HL (480 μmol photons m⁻² s⁻¹) in either TAP (containing acetate as a carbon source) or HS medium (no additional carbon source in the media). Each growth condition was tested in presence (+N) or absence (−N) of nitrogen by depriving the cells for 48 h in a medium without NH₄Cl. WT (4A⁺) and npq4lhcsr1 mutant. 40 μg of total protein were loaded in each lane for PsbS detection, and 5 μg were loaded for LHCSR, D1, and Histone H3 detection. D1 and H3 were used as the protein loading control.

C. reinhardtii npq4lhcsr1 cells were grown in photoautotrophic media under LL under different carbon regimes. For the purpose of this study, these regimes are defined as low CO₂ (no additional CO₂ input into the media), ambient CO₂ (supplied by air bubbling in the media), and high CO₂ (bubbling with air containing 5% CO₂). After reaching an exponential phase, cultures were transferred to HL, and the accumulation of PsbS and other proteins was analyzed by immunoblotting at different time points after HL illumination (Fig. 3).

Expression of PsbS was induced in WT and npq4lhcsr1 mutant cells within 1 h after transfer of cells to HL, independent of the CO₂ concentration in the medium (Fig. 3). However, the PsbS content did not remain at a constant level but decreased at longer HL exposure time, and the dynamics of PsbS accumulation showed a pronounced dependence on the CO₂ concentrations (Fig. 3). In WT and npq4lhcsr1 cells grown under low CO₂, PsbS expression reached maximum levels after ~10 h and decreased to lower levels after 48 h of HL exposure (Fig. 3, A and B). At ambient CO₂ concentrations, maximum PsbS levels accumulated between 1 and 6 h after HL illumination. In WT cells, PsbS disappeared completely after 24 h, whereas low levels of PsbS were retained up to 48 h in npq4lhcsr1 mutant cells (Fig. 3, C and D). Growth at high CO₂ concentrations, however, led to a pronounced accumulation of PsbS after 1 h of HL exposure and complete degradation at longer illumination times in both WT and mutant cells (Fig. 3, E and F).

Similarly, the expression of LHCSR proteins in WT cells was regulated by light and carbon availability. In low CO₂-grown cells, LHCSR proteins were already expressed under LL growth, and their expression levels increased only slightly upon HL exposure (Fig. 3A). With increasing CO₂, the expression of the protein was activated upon transfer to HL (Fig. 3, C and E). LHCSR proteins accumulated gradually in WT cells grown at ambient CO₂ (Fig. 3C) but were degraded after 10 h of HL in high CO₂ grown cells (Fig. 3E). Remarkably, the expression of the CAH3 protein, a carbonic anhydride located in the thylakoid lumen known to be essential for the carbon-concentrating mechanism under low CO₂, was also expressed in cells under high CO₂ growth but degraded after 24 h of HL exposure (Fig. 3, E and F).

In contrast, the protein level of PSII core subunit D1 appeared relatively constant (Fig. 3). NPQ Activation during High Light Acclimation at Different CO₂ Availability—Because both PsbS and LHCSR had similar expression patterns in response to HL exposure at different CO₂ concentrations, we further investigated whether these changes correlated with the quenching capacity during HL acclimation. The activation of NPQ was strongly dependent on CO₂ availability (Fig. 4) and required the accumulation of LHCSR proteins (Fig. 4, B, D, and F), emphasizing the essential role of these proteins for qE in *C. reinhardtii* (8). In WT, NPQ induction was inversely proportional to CO₂ availability: NPQ induction under HL conditions was fully suppressed in the presence of 5% CO₂ (Fig. 4E). At low CO₂ (Fig. 3A) and ambient CO₂ (Fig. 4C), however, activation of NPQ capacity was induced after 6 h of HL exposure. The rapid dark reversibility of this NPQ indicates that predominantly the pH-dependent qE quenching was activated under these conditions (39). The small fraction of slowly inducible/relaxing NPQ, however, was rather reduced upon HL acclimation. These changes occurred independent of the CO₂ availability and the presence of LHCSR or PsbS and thus likely reflect a general acclimation process. Interestingly, the gradual increase in qE capacity in low and ambient CO₂-grown cells correlated with the time frame for PsbS accumulation (Fig. 3, A and C). However, the full induction of NPQ was delayed compared with peak expression of PsbS, and maximum PsbS levels were reached earlier at ambient CO₂ compared with low CO₂ conditions (Fig. 3, A and C). This suggests that the PsbS protein might be either directly involved in the activation of qE or in other responses required for HL acclimation of cells, especially under limited CO₂ availability, and thus limited photosynthetic capacity. Nonetheless, PsbS could not compensate and/or complement the function of LHCSR.
because no NPQ induction was observed in the npq4lhcsr1 mutant under conditions leading to PsbS expression (Figs. 3 and 4). Therefore, PsbS was not sufficient for qE induction in the absence of LHCSR proteins.

**NPQ Activation and LHCSR Accumulation in PsbS Knockdown Lines**—To further investigate the possible role of PsbS, we studied the activation of NPQ in cells with reduced PsbS expression. Using artificial micro RNA (amiRNA), several independent lines with reduced PsbS expression were identified. NPQ activation in cells grown at ambient CO2 was determined in two of these lines (1–2 and 1–6) after transfer of cells from LL to HL (Fig. 5). Strikingly, the reduction of PsbS accumulation (Fig. 5A) led to a pronounced reduction of the NPQ capacity with a slightly more pronounced reduction in line 1–2, which showed lower PsbS accumulation (Fig. 5B). This implies that the accumulation of PsbS to normal levels is essential to induce the full NPQ capacity upon HL acclimation. The knockdown of PsbS accumulation was accompanied by a strong reduction in the LHCSR content (Fig. 5A), indicating that the reduced NPQ capacity is related to a reduced amount of LHCSR3. The accumulation of high LHCSR levels appears to depend on the accumulation of PsbS, suggesting that PsbS is a prerequisite for the stable accumulation of LHCSR3.

**Localization of PsbS**—The reduction of both the NPQ capacity and LHCSR3 accumulation in PsbS knockdown lines indicates a function of PsbS in thylakoid membranes. However, earlier work showed that overexpressed PsbS did not localize to thylakoid membranes (27). We investigated whether native PsbS accumulates in thylakoid membranes and whether LHCSR3 and PsbS show a similar distribution among the protein complexes. For that, we isolated thylakoid membranes from LL- and HL-acclimated cells and determined the distribution of PsbS and LHCSR after separating protein complexes under native conditions by sucrose gradient ultracentrifugation (Fig. 6). Indeed, the PsbS protein was detected in isolated thylakoids from WT cells exposed to HL, but PsbS and LHCSR showed a different distribution among the isolated fractions. Most of the PsbS co-migrated with PSII core-enriched protein fraction B3, although weak bands were also detectable for PsbS in other bands, whereas LHCSR was found predominantly in fraction B1 (Fig. 6, B and C). This suggests that PsbS is associated with PSII core complexes, in contrast to LHCSR3, which migrates predominantly in band B1 that lacks PSII. It should be noted, however, that also very faint bands for LHCSR3 were visible in fractions B3 and B4, in accordance with the previously reported association of a small fraction of LHCSR3 with PSII-
LHII and PSI-LHCI supercomplexes (40). Conversely, in HL-acclimated qE-deficient cells (npq4lhcsr1), PsbS was mainly co-migrating with light-harvesting proteins (Fig. 6C). The differential PsbS distribution among complexes in cells with and without quenching capacity suggests that changes in the localization of PsbS might be related to the rearrangement of proteins during qE activation upon HL exposure. It is further worth noting that the HL-induced shift in the distribution of the PSII antenna protein Lhcbm5 toward the PSII core-enriched fraction B3 was only detectable in WT cells but not in HL-acclimated npq4lhcsr1 cells (Fig. 6C). This suggests that a structural change involving the organization of the PSII-LHII supercomplexes occurred in the HL-acclimated state in WT and that this change was absent in the mutant lacking LHCSR proteins.

Discussion

The pH-regulated qE mechanism of energy dissipation in C. reinhardtii is controlled by the LHCSR3 protein (8) contrary to land plants; in mosses this function is performed by both PsbS and LHCSR (12, 13) and in vascular plants only by PsbS (18). In this work, we presented evidence that the PsbS protein in C. reinhardtii is a HL-induced protein that regulates the activation of the LHCSR-dependent qE capacity but is not sufficient for inducing qE in the absence of LHCSR proteins.

The qE response (induction and capacity) of C. reinhardtii is regulated at different levels. At the transcript level, LHCSR and PSBS genes are up-regulated in response to changes from low to high light (8, 31, 34). The two PSBS genes (PSBS1 and PSBS2) are up-regulated similarly in response to high light stress (32). In the present work this response was observed also at the protein level after a few hours of HL illumination (Figs. 2 and 3). Due to the identical amino acid sequence of PSBS1 and PSBS2, the antibody that we generated detects both proteins. In addition to a shift in light intensity, the availability of CO2 (and hence the capacity of photosynthesis) plays an important role in the regulation of the qE machinery. In the absence of HL, LHCSR protein accumulated under low CO2 conditions (Figs. 2A and 3A), which is likely due to an EEC motif (enhancer element of low CO2-inducible genes) in its promoter region (34, 41). Furthermore, the LL intensity of 30 μmol photons m−2 s−1 used here may already saturate photosynthesis at limiting CO2 availability. PsbS protein level increased during HL under all CO2 conditions tested (Figs. 2 and 3), but the sustained accumulation of PsbS protein during HL was observed only under low CO2 conditions (Fig. 3). The promoter regions of both PSBS genes contain EECs (Fig. 7), and thus both genes are likely to be transcriptionally induced under our low CO2 conditions, although it does not exclude other possible modes of regulation. Additionally, the accumulation of LHCSR requires Ca2+ (42). However, whether or not HL-induced accumulation of PsbS is also Ca2+-dependent remains to be elucidated.

The analysis of qE induction curves along with the accumulation of PsbS and LHCSR under different growth conditions identified specific requirements for qE quenching in C. reinhardtii. The following important conclusions regarding the induction of qE in C. reinhardtii can be drawn. (i) The presence of LHCSR in LL-grown cells before the onset of HL illumination (Figs. 2A and 3A) is not sufficient for qE induction (Fig. 4). Consequently, further acclimation processes are required for establishing a high qE capacity. (ii) The presence of both PsbS and LHCSR3 after 1 h of HL exposure in high-CO2-grown cells (Fig. 3E) is also not sufficient for qE induction (Fig. 4E). This result together with the rapid degradation of both PsbS and LHCSR during longer illumination periods suggests that qE is generally not activated at high CO2 concentrations. (iii) The presence of PsbS is not sufficient to induce any qE in the absence of LHCSR, even after longer periods of HL acclimation (Figs. 3, B and D, and 4, B and D). This underscores that LHCSR is essential for qE. (iv) PsbS accumulates only transiently to maximal levels during HL acclimation and undergoes degradation before induction of full qE capacity (Fig. 3A and C, 4A, and C). Hence, the accumulation of both proteins, LHCSR and PsbS, is not sufficient to induce the full NPQ capacity, indicating a critical role of PsbS during the establishment of high qE capacity. (v) Knockdown of PsbS expression leads to a strong reduction of both LHCSR accumulation and NPQ capacity (Fig. 5), suggesting that PsbS controls processes that are essential to establish a specific interaction of LHCSR with PSI required for qE (and not mere stabilization of LHCSR because LHCSR was detected in multiple conditions in
which PsbS was absent; Fig. 3, A–C). (vi) Complete degradation of PsbS is not required for maintaining a high qE capacity, as high qE can be induced in the presence of low PsbS levels after 48 h of HL acclimation under low CO₂ (Figs. 3A and 4A). This implies that PsbS does not interfere with the quenching site.

Similar to LHCSR, the expression of PsbS at the transcript and protein levels is regulated by light and CO₂ availability.
PsbS Function in C. reinhardtii

However, PsbS accumulated only transiently during the establishment of qE capacity (Fig. 3). Together with the strongly reduced LHCSR accumulation and NPQ capacity in cells with reduced PsbS expression (Fig. 5), these characteristics are consistent with the hypothesis that PsbS is essential for conformational changes in the antenna of PSII that are required for the binding of LHCSR3 at a specific qE-quenching site. In general, such a function of PsbS resembles that in vascular plants despite the fact that PsbS is constitutively expressed in vascular plants. Our recent finding that PsbS from *C. reinhardtii* can increase NPQ capacity when transiently expressed in the plant *Nicotiana benthamiana* supports the concept of a similar function for PsbS from green algae and plants. In contrast to overexpressed PsbS (27), native PsbS localized to the thylakoid membrane (Fig. 6). The mismatched localization of overexpressed PsbS is likely the result of the specific growth or expression conditions used in the former study (27).

In *C. reinhardtii*, two qE quenching sites have been proposed to be involved in qE: one in the minor light-harvesting complex and another in aggregated LHCCI trimers, detached from PSII (43), similar to the situation in *A. thaliana* (44). Within the thylakoid membrane, PsbS was found to co-migrate with PSII core proteins and not with LHCSR in HL-acclimated cells (Fig. 6C), suggesting an interaction of PsbS with the PSII reaction center upon HL acclimation. However, in the absence of LHCSR proteins (*npq4lhcsr1*), this affinity for PSII was reduced (Fig. 6C). Although PsbS did not co-localize with LHCSR proteins in the light (Fig. 6C), its affinity toward PSII in a quenched state could indicate that PsbS is involved in the detachment of LHCCI proteins from PSII, which might be essential for subsequent LHCSR binding to the LHCs and hence qE induction. Such an interaction of PsbS resembles that in vascular plants where an interaction of PsbS with LHCCI and PSII reactions center proteins has been proven (23).

The strongly decreased levels of LHCSR proteins in *PSBS* knockdown lines (Fig. 5) indicates that the stable accumulation of LHCSR3 depends on the presence or action of PsbS. Whether a PsbS-controlled reorganization of the PSII antenna, such as the detachment of LHCCI proteins, promotes binding of LHCSR3 to PSII or whether a direct interaction of PsbS with LHCSR3 is required for LHCSR3 binding to PSII remains to be clarified. However, the following observations, (i) the presumably stoichiometric amount of PsbS accumulating upon HL acclimation, (ii) the delayed accumulation of LHCSR3 protein levels compared with that of PsbS (Fig. 3), and (iii) the different localization of PsbS and LHCSR proteins, argue against a direct interaction of PsbS and LHCSR3 upon activation of a high NPQ capacity. It is, therefore, conceivable that PsbS interacts with LHCCI and PSII reaction center proteins and thereby reorganizes the PSII antenna to promote binding of LHCSR3. It is tempting to speculate that PsbS induces the detachment/reorganization of specific PSII antenna proteins, which prevent binding of LHCSR3 to qE-specific sites. Such an action of PsbS would only be required transiently because the PsbS is no longer necessary once a high NPQ capacity is established (Figs. 3 and 4). This implies that the PsbS-induced reorganization of the PSII antenna is stable in the HL-acclimated state. Recently, the PSI subunit PsbR has been shown to be required for the binding of LHCSR3 to PSII upon HL acclimation (40). However, reduction of PsbR accumulation did not lead to reduced LHCSR3 accumulation (40). Presumably, functional binding of LHCSR3 to PSII is controlled by several factors, but PsbS is essential for accumulation of LHCSR3.

Our data have further important implications on the evolution of qE quenching in oxygenic photosynthetic organisms. In green algae, PsbS already has (as in vascular plants) an essential function in promoting conformational changes for qE activation, but the function of pH regulation of qE is restricted to LHCSR3. At early stages of land colonization, as represented by the moss *P. patens*, PsbS gained an additional function as an independent pH regulator of qE, whereas the function of LHCSR3 became independent of PsbS, so that both proteins can act independently in qE regulation. At late stages of land colonization, as in vascular plants, the function of LHCSR3 was completely lost and PsbS gained the full capacity as pH regulator of qE quenching. Future work is required to understand the functional switch of both PsbS and LHCSR3 upon land colonization.

**Experimental Procedures**

**Cells and Growth Conditions**—*C. reinhardtii* wild-type strain 4A+ (137c genetic background) and the double mutant *npq4lhcsr1* (11) lacking the LHCSR3 and LHCSR1 proteins (encoded by *LHCSR3.1, LHCSR3.2*, and *LHCSR1*) were grown in batch cultures at 23 °C and constantly stirred on a shaker at 112 rpm. Light regimes were defined as low light (LL; 30 μmol photons m⁻² s⁻¹) and high light (HL; 480 μmol photons m⁻² s⁻¹).

The amiRNA target sequence, TCCAAGCTCTTAGGGGGC, was designed to silence both *PSBS1* and *PSBS2* using Web MicroRNA Designer (WMD3). The synthesized oligo was inserted into pChlamiRNA3int (45) and transformed into 4A+ (46). The resulting paromomycin-resistant strains were

7 Leonelli, L., Erickson, E., Lyska, D., and Niyogi, K. K. (2016) Transient expression in *Nicotiana benthamiana* for rapid functional analysis of genes involved in non-photochemical quenching and carotenoid biosynthesis. *Plant J.*, in press.
screened by immunoblotting of PsbS in LL- and HL-grown cells.

**Nitrogen Starvation Experiments**—WT 4A + npq4lhcsr1 cells were grown in a 50-ml preculture in TAP (47) or HS (48) media containing 7.2 mM and 9.4 mM NH₄Cl, respectively (+N media). Cells were grown under constant LL (30 μmol photons m⁻² s⁻¹) for 3–5 days until the culture reached a density of 3–5 × 10⁶ cells/ml. 25 ml of the preculture were used to inoculate 100 ml of the same media, and cultures were grown under LL or HL. Once the cultures reached 5 × 10⁶ cells/ml, nitrogen starvation was induced by transferring the cells to media without any NH₄Cl (TAP or HS, -N), and cells were grown for the next 48 h under the respective light regime. Subsequently, cells were harvested by centrifugation at 3,850 × g, frozen in liquid nitrogen, and stored at −20 °C for further analysis.

**Growth at Different Carbon Availabilities**—For each strain, cells were grown in three 50-ml HS media precultures under LL for 5 days. Each preculture was used to inoculate 700 ml of HS media with either no additional air input (no CO₂) or bubbling with air (ambient CO₂ ≈ 0.035–0.04%) or air enriched with 5% CO₂ (high CO₂). Cells were grown under LL until reaching a density of 5 × 10⁶ cells/ml and were subsequently transferred to HL. For protein analysis, 100 ml of sample were taken after 0, 1, 6, 10, 24, and 48 h of HL exposure. After harvesting the samples, 100 ml of fresh HS media was added to each culture. The same procedure was used when sampling cells after the 0-, 30-, 60-, 120-, 180-, and 360-min transfer to HL. In this case, cells were grown in HS without additional CO₂. All samples were harvested and stored for further analysis as described for the nitrogen starvation experiments.

**Expression and Purification of Recombinant PsbS Protein**—Recombinant PsbS (GenBank™ ID 5715134) was cloned into the pET28a (+) vector (Novagen), kindly provided by Katrin Gärtnert (Michigan State University), as described previously (27). Recombinant PsbS was overexpressed in BL21 gold E. coli cells, extracted by French press treatment, and purified by affinity chromatography (nickel-nitrilotriacetic acid column, Bio-Rad). Protein concentrations were quantified using the DCTM protein assay (Bio-Rad). Cells were disrupted by pressure in a French press (1000 p.s.i., 4 °C) 3 times and centrifuged for 10 min at 12,000 × g, 4 °C. The pellet was resuspended with a paint brush in 15 ml of 5 mM MgCl₂, centrifuged again, resuspended in 200–250-μl aliquots with 5 mM MgCl₂, frozen in liquid N₂, and stored at −20 °C.

**Sucrose Gradient Ultracentrifugation**—Protein complexes of the thylakoid membrane were separated by sucrose density gradient centrifugation as described by Tokutsu et al. (51) with some modifications. Stacked membranes were unstacked by adding 1 volume of 5 mM EDTA and centrifuged for 1 min at 17,000 × g at 4 °C. Unstacked membranes were solubilized in a medium containing 25 mM MES, pH 6.5, and 1% n-dodecyl-α-d-maltoside at a final concentration of 0.4 mg chlorophyll/ml. Insoluble material was removed by centrifugation at 17000 × g for 10 min. Solubilized thylakoids corresponding to 200 μg of chlorophyll were loaded onto a discontinuous sucrose gradient (0.1/0.4/0.7/1.0/1.3 M sucrose, 25 mM MES, pH 6.5, 1 mM betaine, 0.02% n-dodecyl-α-d-maltoside) and fractionated by ultracentrifugation for 16 h at 130,000 × g at 4 °C. After separation, each band was collected, and half of the volume was used to quantify the pigment content by reverse HPLC (52), whereas the other half was used for protein analysis. Proteins corresponding to 2 μg of chlorophyll (and 25 μg of chlorophyll for PsbS detection) were separated on a NuPAGE® Novex® 10% Bis-Tris gel (Life Sciences) and visualized with a Sypro® Ruby protein gel staining according to the manufacturer's protocol. Immunological detection was performed as described.

**Chlorophyll Fluorescence Measurements**—Chlorophyll fluorescence was measured using a JTS-10 spectrometer (Bio Logic SAS, Claix, France). A volume corresponding to 2 × 10⁶ cells was dark-adapted under stirring at ambient CO₂ for 15 min and then filtered onto a glass fiber filter (PALL Corp.). The filter was fixed in a leaf cuvette, and samples were exposed for 5 min to far red light (400 μmol photons m⁻² s⁻¹) followed by 15 min of illumination at 940 μmol photons m⁻² s⁻¹ of red actinic light and 6 min of incubation in far red light. Saturation pulses (red light, 7900 μmol photons m⁻² s⁻¹) were applied every 60 s. NPQ was calculated as (Fm/Fm') − 1, with Fm and Fm' being the maximum fluorescence in the dark- and light-adapted state, respectively (53).

**Cell Counting**—Cells in a 1-ml culture were fixed with 20 μl of 0.25% iodine (w/v in ethanol), and the number of cells/ml was calculated using a Thoma cell counting chamber (Marienfeld, Lauda-Königshofen, Germany).
PsbS Function in *C. reinhardtii*

**Author Contributions**—V. C.-G., S. W., K. K. N., and P. J. designed the research. V. C.-G., P. R., K. G., A. G., and T. B. T. performed the experiments. V. C.-G., P. R., S. W., K. K. N., and P. J. analyzed data. V. C.-G. and P. J. wrote the manuscript.

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