

# *Physcomitrella patens* mutants affected on heat dissipation clarify the evolution of photoprotection mechanisms upon land colonization

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Light is the source of energy for photosynthetic organisms; when in excess, however, it also drives the formation of reactive oxygen species and, consequently, photoinhibition. Plants and algae have evolved mechanisms to regulate light harvesting efficiency in response to variable light intensity so as to avoid oxidative damage. Nonphotochemical quenching (NPQ) consists of the rapid dissipation of excess excitation energy as heat. Although widespread among oxygenic photosynthetic organisms, NPQ shows important differences in its machinery. In land plants, such as *Arabidopsis thaliana*, NPQ depends on the presence of PSBS, whereas in the green alga *Chlamydomonas reinhardtii* it requires a different protein called LHCSR. In this work, we show that both proteins are present in the moss *Physcomitrella patens*. By generating KO mutants lacking PSBS and/or LHCSR, we also demonstrate that both gene products are active in NPQ. Plants lacking both proteins are more susceptible to high light stress than WT, implying that they are active in photoprotection. These results suggest that NPQ is a fundamental mechanism for survival in excess light and that upon land colonization, photosynthetic organisms evolved a unique mechanism for excess energy dissipation before losing the ancestral one found in algae.

LHCSR | nonphotochemical quenching | photosystem | plants evolution | PSBS

Sunlight provides energy supporting the life of photosynthetic organisms but also leads to the formation of reactive oxygen species when in excess (1, 2). During early Devonian, when plants first colonized terrestrial habitats, they underwent no competition by other organisms. However, they had to adapt to harsher physicochemical conditions than in the original water ecosystem (3) because in the atmosphere concentration of oxygen, an inhibitor of photosynthesis, is higher (4) and concentration of carbon dioxide, the final acceptor of electrons extracted from water by photosystems, is lower. Moreover, the sessile form of life acquired on land prevented escape from rapid changes in light intensity by swimming deeper, a behavior typical of algae (5). The combination of these conditions makes it more likely that light is harvested in excess with respect to the maximal rate of photochemical reactions, and a fast and efficient photoprotection response is essential for survival.

The fastest response to high light stress is provided by non-photochemical quenching (NPQ), which consists of the thermal dissipation of the chlorophyll excited singlet states (<sup>1</sup>Chl\*) (6–8). NPQ has two major components: energy quenching (qE), which is activated within seconds on an increase in light intensity, and inhibitory quenching, which is slower and relaxes within 1–2 h in the dark (7, 9). In vascular plants, qE activation requires PSBS, a protein homologous to light harvesting antenna subunits of photosystems (Lhc) (10), which is activated by the accumulation of protons in the chloroplast lumen and the protonation of two glutamate residues (11). Activated PSBS induces a decrease in excited state lifetime in the pigment-binding subunits of the antenna system consequent to a reorganization of photosystem subunits in the thylakoid membrane (12–14). NPQ was proven to

be particularly important in field conditions, wherein plants lacking PSBS are rapidly counterselected (8).

The green alga *Chlamydomonas reinhardtii* shows different requirements for NPQ activation, and the process is induced by acclimation to high light, whereas it is constitutive in plants. Also, this green alga does not need PSBS (15–17) but a distinct Lhc-like protein, called LHCSR (or Li818), implying important differences in the mechanism for activation of heat dissipation (17). LHCSR has been found in many taxa, such as brown and green algae (18), but not in vascular plants. Conversely, although genes encoding PSBS have been identified in the genome (19), the protein itself has not been detected in algae to date (16), consistent with their NPQ being dependent on LHCSR. Further evidence that NPQ activation is different in algae and vascular plants (20) is provided by the lack in the former of Lhcb6, an antenna system component involved in PSBS-dependent NPQ activation of plants (12, 21).

The organization of photosystems is very similar in vascular plants and green algae but for the absence in the latter of Lhcb6 and Lhcb3 (18, 20). Grana stacks only developed in less ancient taxa of the Streptophyta line (Coleochaetales and Charales) (22, 23), whereas other algae, at most, show stacking between two thylakoid membranes.

Studying organisms that diverged from the green lineage leading to vascular plants early after land colonization helps us to understand how photosynthetic organisms adapted to the challenges of different environmental conditions. The moss *Physcomitrella patens* is a valuable choice as a model organism because its genome has been completely sequenced and tools are available for its genetic manipulation (3, 24). Early studies on the organization of photosynthetic genes showed that *P. patens* is the only example among plants wherein both LHCSR and PSBS genes are present (3, 18, 20). In this work, we show that both of these genes are expressed and the corresponding polypeptides are accumulated in the thylakoid membranes. By generating specific KO mutants for each of the two LHCSR genes and PSBS in the *Physcomitrella* genome, we show that both proteins are active in promoting NPQ and contribute to photoprotection under excess light conditions. These results suggest that the PSBS-dependent NPQ of plants evolved before the LHCSR-based mechanism typical of algae was lost. The latter disappeared at later stages of plant evolution, when the newly evolved PSBS-dependent mechanism ensured a sufficient level of photoprotection.

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## Results

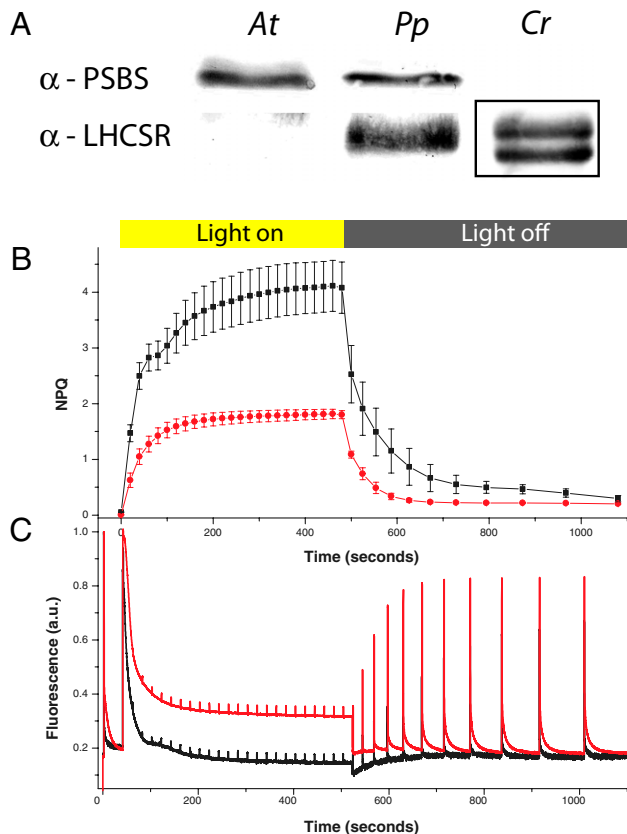
***P. patens* Expresses Both LHCSR and PSBS and Shows High NPQ.** NPQ in the green alga *C. reinhardtii* relies on the presence of LHCSR. This group of Lhc-like proteins has been found in several algae but not in land plants, with the remarkable exception of *P. patens*, a bryophyte that diverged from vascular plants around 400 million years ago, early after land colonization (3, 25). A search of The Institute for Genomic Research (TIGR) sequence databases allowed identification of several ESTs encoding LHCSR and PSBS proteins in another bryophyte, *Tortula ruralis*, as well (TA794\_38588 and CN203629, respectively), suggesting that this is not a unique property of *P. patens*.

Finding genome sequences does not imply that the corresponding polypeptides are expressed or that they play the expected function, as shown in the case of PSBS in many green algae (16). We therefore searched for LHCSR and PSBS polypeptides in *P. patens* thylakoids by using specific antibody probes. We detected specific signals at the expected molecular mass for *P. patens* LHCSR (around 23 kDa) (20) and PSBS (22 kDa), showing that both proteins are actively expressed and accumulate in the membrane of thylakoids (Fig. 1*A*). A search in *Arabidopsis* and *Chla-*

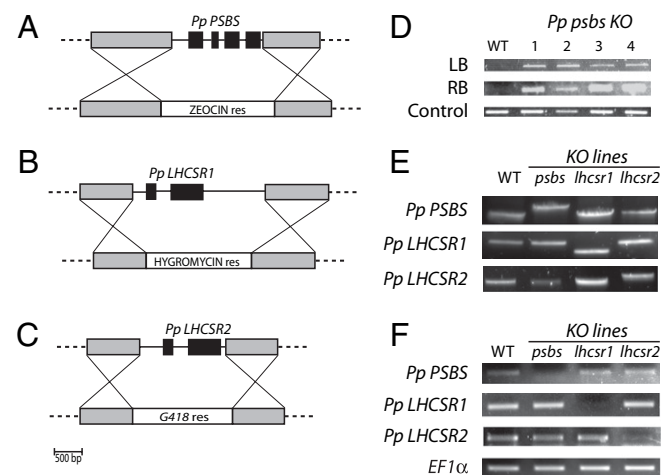
*mydomonas* extracts yielded a signal for PSBS and LHCSR only, respectively. In *Chlamydomonas*, LHCSR is detected only in extracts from high light-acclimated cells, consistent with the recent report that high light acclimation is needed for its accumulation and induction of NPQ activity (17) and at variance with *P. patens*, in which it is constitutively accumulated.

Fig. 1*B* shows that NPQ activation in response to illumination with strong actinic light of dark-adapted plants is higher in *P. patens* with respect to *Arabidopsis*. Consistently, the decline of maximal fluorescence yield ( $F_m'$ ), a parameter closely reflecting the lifetime of chlorophyll (Chl) a singlet excited states in the antenna compartment, is faster and the final intensity is lower in the moss with respect to the vascular plant (Fig. 1*C*). It should be noted that actinic light intensity during the measurement was chosen to be just sufficient to saturate photosynthesis to ensure maximal NPQ amplitude in both organisms. Despite using lower actinic light intensity (800 vs. 1,200  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ), capacity of heat dissipation was higher in *P. patens* than in *Arabidopsis thaliana*. Consistently, the stronger NPQ in mosses was observed for a large range of actinic lights (Fig. S1), confirming that the capacity of dissipating excitation energy as heat was higher in the moss with respect to the plant.

**Targeted KO of PSBS, LHCSR1, and LHCSR2.** It is tempting to hypothesize that the faster and stronger photoprotection found in mosses relies on the action of both PSBS-dependent and LHCSR-dependent mechanisms of energy dissipation. To assess if this was indeed the case, we produced specific KO mosses depleted in the three genes encoding putative NPQ triggering polypeptides. *P. patens* is a very valuable organism for gene KO because it performs homologous recombination at high efficiency (24). Disruption constructs for the three genes were created to substitute the entire coding sequences with selection cassettes, as schematized in Fig. 2 *A–C*. PEG-mediated pro-



**Fig. 1.** Comparison of NPQ *A. thaliana*, *P. patens*, and *C. reinhardtii*. (*A*) Western blotting against NPQ involved polypeptides, PSBS, and LHCSR in *Arabidopsis* (At), *Physcomitrella* (Pp), and *Chlamydomonas* (Cr). Thylakoids (1  $\mu\text{g}$  of Chl) were loaded in all cases. In the case of Cr, thylakoids were isolated from high light-grown cells; here, two LHCSR bands are recognized as in the study by Peers et al. (17). Bands are squared because LHCSR has a larger molecular mass in Cr with respect to Pp (29 vs. 23 kDa). Anti-LHCSR antibody recognizes other proteins with lower specificity (likely Lhc proteins) at different molecular masses in both At and Pp. NPQ (*B*) and fluorescence (*C*) kinetics were measured for Pp (black) compared with At (red). Actinic light intensities were shown to saturate photosynthetic capacity in both organisms, as shown by the fact that pulses induce a very small peak in fluorescence ( $F_m'$ ), and thus activate maximal NPQ.



**Fig. 2.** KO mutant generation and characterization. Scheme of constructs used for KO generation. Genomic region of *PSBS* (*A*) and *LHCSR* (*B* and *C*) genes is schematized, with exons shown in black. Gray boxes represent the genomic regions exploited for homologous recombination. Below are shown the constructs for homologous recombination: genes for antibiotic resistance are located between regions homologous to the genome (all primers used for mutagenesis are reported in Table S1). (*D*) Example of verification of DNA insertion in the genome by amplification of the right and left borders. An example of four independent lines of *Psbs* KO is shown. (*E*) Amplification of genomic DNA using primers external to the target recombination region. Mutants carrying a single insertion are identified by the different size of the amplified band with respect to WT. Amplificates were also sequenced to verify the insertion in the correct position. (*F*) Evaluation of *PSBS* and *LHCSR* gene expression assessed by RT-PCR in WT and different mutant lines. Elongation Factor-1 alpha (*EF1 $\alpha$* ) is also reported as a control.

toplast transformation led to the isolation of stably resistant colonies that were characterized by PCR to verify the presence of the insertion at the expected target site. A first set of PCR assays on genomic DNA was performed to amplify both left and right borders of the inserted cassette (an example of amplicates for selected lines of *psbs* mutants is reported in Fig. 2D). At least four independent clones with the insertion in the correct position were isolated and retained for further characterization.

During transformation in *Physcomitrella*, multiple insertions at the same site might occur (24, 26, 27). To select single copy insertions, we used a PCR assay with primers annealing to the genomic sequences at either side of the insertion region. PCR amplifications thus are possible only in the case of a single insertion, whereas for multiple insertions, fragments are too large to be efficiently amplified (Fig. 2E). All PCR fragments were sequenced for further control, and we finally retained at least two independent single insertion lines for each transformation. The suppression of gene expression was confirmed by RT-PCR with specific primers: cDNAs were all present in WT and lost in the corresponding mutants (Fig. 2F).

Single-insertion mutants were grown, and 10-day-old plants were harvested and used for thylakoid purification. When analyzed by Western blotting, no signal for PSBS was detected in *psbs* KO (Fig. 3A). KO for *LHCSR* showed that most of the immunoblotting signal was lost in *lhcsr1* KO, whereas protein level was substantially unaffected in *lhcsr2* KO. Red Ponceau staining and anti-CP43 antibody were routinely used to verify equal loading for all samples (Fig. 3A). In all mutants, we observed no significant alterations in Chl *a/b* and Chl/carotenoid

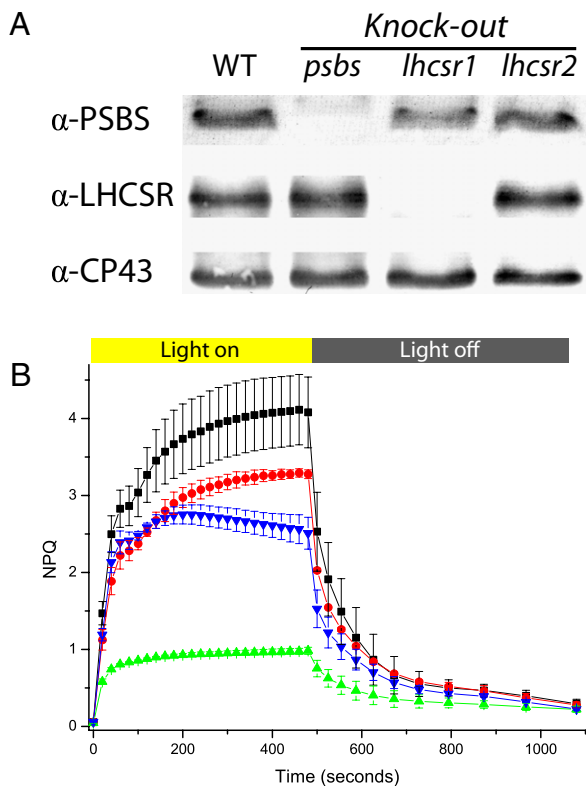
content (Table 1), which indicates that mutations do not affect the composition of the photosynthetic apparatus, which is thus comparable in all mutants. The maintenance of photosystem organization was also confirmed by measuring the functional kinetics in plants treated with dichloromethylurea (DCMU) (Fig. S2). Also, the quantum yield of photosystem II (PSII) in dark-adapted samples was unaffected: All plants showed a variable over maximal fluorescence (Fv/Fm) value of  $0.80 \pm 0.03$  after 10 days of growth in control light conditions.

When NPQ kinetics were recorded instead, KO plants showed large differences with respect to WT (Fig. 3B). The stronger phenotype was observed in the case of *lhcsr1* KO, followed by *psbs* KO and *lhcsr2* KO. This pattern is consistent with the Western blotting analysis shown in Fig. 3A: most LHCSR protein immunodetected in WT is synthesized by the *PpLHCSR1* gene, and the other isoform is expressed at lower levels, explaining the small phenotype of *lhcsr2* KO. It should be pointed out that by overloading *lhcsr1* KO thylakoids, we were able to detect LHCSR2 by Western blotting, whose content is below the detection limit in Fig. 3, as shown in Fig. S3. Although the antibody might bind to the two isoforms with different affinity, these results strongly suggest that there is no major compensatory increase of LHCSR2 in the absence of LHCSR1.

It is interesting to observe that all KO mutants are still competent in NPQ, with their activity being comparable to NPQ levels measured in WT *Arabidopsis* (Fig. S4). Furthermore, the largest fraction of NPQ can be attributed to qE, the component dependent on low luminal pH, from its fast kinetics of relaxation and sensitivity to nigericin. This is different from the case of *Arabidopsis*, in which residual NPQ in the PSBS-less mutant *npq4* is very small and slow in both rise and relaxation (10).

We report data from only one line for each genotype, but results have been replicated for all other selected lines. Also, we did not observe any significant difference in NPQ amplitude or PSBS/LHCSR protein accumulation between mutants having single or multiple insertions, suggesting that the effect of inserting multiple copies of DNA is not relevant for the observed phenotype (an example is shown in Fig. S5). In all cases, there was a strict correlation between the recombination event shown by PCR assay, the absence of the protein on Western blotting, and the amplitude of the NPQ phenotype, implying that the low NPQ phenotype was attributable to the KO mutations (Fig. S5). Moreover, transformations using two different plasmids carrying either zeocin or neomycin resistance were tested in the case of PSBS. In both cases, the phenotype was the same, confirming that effect of the presence/absence of PSBS on NPQ is much stronger than other possible secondary effects (Fig. S6).

**Double and Triple KO Mutants.** Once the role of each gene in NPQ was assessed, we performed further transformation on mutant backgrounds to obtain double and triple KO mutants. The presence of the mutation was always verified by PCR and Western



**Fig. 3.** *P. patens psbs* and *lhcsr* KO phenotypes. (A) Western blotting using antibodies against LHCSR and PSBS. Western blotting against CP43 is also shown as a loading control. Thylakoids were loaded in all cases, 0.5  $\mu$ g of Chl for anti-PSBS and 0.3  $\mu$ g of Chl for all the others. (B) NPQ kinetics of selected lines. Curves for WT and *psbs* KO, *lhcsr1* KO, and *lhcsr2* KO are shown in black, blue, green, and red, respectively. Averages and SD are calculated from at least five independent measures.

**Table 1.** Pigment-binding properties of isolated mutants

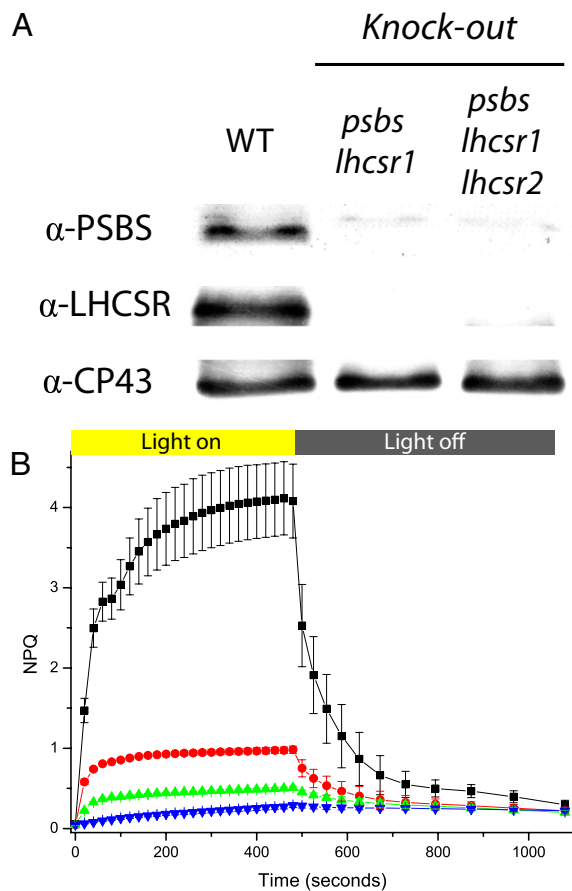
	WT	<i>psbs</i> KO	<i>lhcsr1</i> KO	<i>lhcsr2</i> KO
Chl <i>a/b</i>	2.56	2.58	2.52	2.60
Chl/Car	4.33	4.25	4.60	4.43

	WT	<i>psbs lhcsr1</i> KO	<i>psbs lhcsr1 lhcsr2</i> KO
Chl <i>a/b</i>	2.56	2.42	2.57
Chl/Car	4.33	4.52	4.29

Pigment-binding properties of single and multiple mutants depleted in PSBS, LHCSR1, and LHCSR2, respectively. Chl *a/b* and Chl/carotenoids (Car) ratios are reported. SD is below 0.1 in the case of Chl *a/b* and 0.3 for Chl/Car.

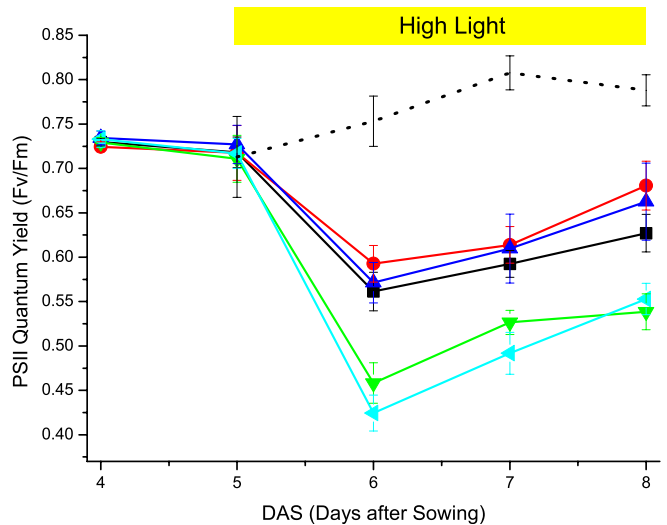




**Fig. 4.** Phenotypes of *P. patens psbs* and *lhcsr* KO double and triple mutants. (A) Western blotting using antibody against LHCSR and PSBS. (B) NPQ kinetics of selected lines. Curves for WT, *lhcsr1* KO, *psbs lhcsr1* KO, and *psbs lhcsr1 lhcsr2* KO are shown in black, red, green, and blue, respectively. Averages and SD are calculated from at least five independent measures.

blotting, as described above (Fig. 4A). The depletion of LHCSR1 in *psbs* KO background causes a further reduction in NPQ. However, this double mutant still retains a residual NPQ having qE properties, which can be attributed to the residual presence of LHCSR2. This hypothesis was verified by isolating triple mutants, where LHCSR2 was also missing. NPQ in the triple mutant was very low and completely depleted in its fast component, qE (Fig. 4B). In fact, the small residual NPQ was stable during at least 10 min of dark recovery following illumination, as observed in the PSBS-less *npq4* mutant of *Arabidopsis* (10).

**Role of Individual LHCSR and PSBS Gene Products in Protection from High Light Stress.** NPQ is a mechanism known to contribute to resistance to light stress in both higher plants and algae (8, 17, 28). To prove this is also the case in *P. patens*, we treated WT and mutant 5-day-old plants with high light intensities. Fv/Fm was measured every day to monitor PSII photodamage. As shown in Fig. 5, WT plants, on the shift to high light conditions, showed a drastic decrease in PSII efficiency, a clear sign of photoinhibition. PSII efficiency recovers over the following days, suggesting that plants start acclimating to the light conditions. Single KO mutants were similarly affected by the light treatment, whereas the double *psbs lhcsr1* and the triple *psbs lhcsr1 lhcsr2* KO mutants showed a stronger degree of PSII photoinhibition. This is a clear indication that the shift to high light conditions affects plants depleted in both PSBS and LHCSR more drastically. Despite experiencing a stronger photoinhibition, however, even



**Fig. 5.** Photoprotection capacity of NPQ-affected mutants. The photoprotection capacity of the previously described mutants has been compared with WT by treating 5-day-old plants with an additional 3 days of high light. PSII quantum yield, expressed as Fv/Fm, was monitored daily. WT, *psbs* KO, and *lhcsr1* KO are shown in black, red, and blue, respectively, whereas *psbs lhcsr1* and *psbs lhcsr1 lhcsr2* KO double and triple mutants are shown in green and cyan. Values for a WT culture that was kept to control light conditions are also reported in black dots. Data from mutants grown in control conditions were omitted for clarity because they were indistinguishable from data on WT culture.

these plants show partial recovery over the following days, demonstrating that other acclimating mechanisms acting with slower time courses are active in mosses independent of NPQ, as previously reported for plants and algae (29–31). In single KO mutants, phenotype on photoprotection is small, if any, suggesting that PSBS and LHCSR are able to provide a photoprotection capacity similar to WT, even in the absence of the other subunit, in these conditions.

## Discussion

**PSBS and LHCSR Are Active Independently in *P. patens* NPQ.** *Arabidopsis* NPQ is well known to depend on the presence of the PSBS subunit of photosystem II (10, 11). Although the *PSBS* gene is conserved in the genome of some green algae (19), it was never detected as polypeptide (16). On the contrary, in the green alga *C. reinhardtii*, it has been shown recently that NPQ requires LHCSR, a different Lhc-like polypeptide previously called Li818. Genes encoding the LHCSR polypeptide are found in many algae groups, including diatoms (32).

PSBS gene and polypeptide have been identified in several plant species that also show comparable NPQ kinetics, and all data available suggest that NPQ depends on PSBS in vascular plants. Algae are a highly diversified and less characterized group of organisms; thus, we should be cautious before generalizing. Nevertheless, so far, PSBS polypeptide has never been detected in any green alga belonging to Viridiplantae, despite the presence of a corresponding gene in the genome (16). Thus, present knowledge is consistent with the hypothesis that NPQ activation relies on different mechanisms. This was proven to be true for *Chlamydomonas* and *Arabidopsis*, the two model systems for green algae and plants, respectively, which are the subject of the present study. Although the molecular mechanisms of LHCSR action are still unclear, available data in *Arabidopsis* suggest that PSBS acts as a modulator for antenna proteins (12, 21, 33, 34). Among different Lhc proteins, Lhcb3 and Lhcb6 were shown to be specifically involved in PSBS-dependent NPQ activation (12,

21) and are present only in plants. So far, no evidence has been obtained for their presence in algae (20), suggesting that the function of PSBS in algae, if any, is substantially different from that in plants.

*P. patens* is the only plant in which both PSBS and LHCSR gene sequences have been found (20). The report of PSBS and LHCSR EST sequences in *T. ruralis* suggests that they are present in other mosses as well. There is no indication at present that any vascular plants carry LHCSR-encoding sequences in their genomes, which suggests a change in the mechanisms of photoprotection in Viridiplantae during evolution. In this work, we have analyzed the role of LHCSR and PSBS polypeptides in *P. patens* by generating specific KO plants: all mutants showed a decreased NPQ phenotype, implying that both LHCSR and PSBS are active in triggering this photoprotection mechanism.

LHCSR and PSBS NPQ activities in *P. patens* are largely independent; in this study, we have shown that mutants lacking PSBS or most of the LHCSR protein (*psbs* and *lhcsr1* KO, respectively) are still capable of significant NPQ. Although mutual interactions cannot be excluded, their effect appears to be additive, consistent with the observation that in conditions of maximal NPQ, fluorescence quenching in *P. patens* is faster and stronger than in *Arabidopsis* (Fig. 1). Consistently, *lhcsr1* KO plants, missing most of the LHCSR protein, show an NPQ amplitude comparable to that of *Arabidopsis* plants (Fig. S4).

The mutant phenotype in high light stress experiments also supports this hypothesis; we observed strong light sensitivity only when PSBS and LHCSR were both absent (or largely depleted as in the case of *psbs lhcsr1* double KO mutant). When only one protein was present, it was sufficient to activate enough NPQ to provide protection levels equivalent to WT in the experimental conditions tested.

**Evolution of Heat Dissipation Mechanisms.** Presented results allow the proposal that *P. patens* retains the LHCSR-dependent NPQ mechanism found in *Chlamydomonas*, although additionally showing the newly evolved PSBS-dependent mechanism typical of land plants. Together, these mechanisms provide optimal photoprotection of *Physcomitrella* in high light conditions. Our results suggest that the PSBS-dependent mechanism of excess energy dissipation appeared during plant evolution before the ancestral LHCSR-dependent function was lost. This finding highlights the importance of photoprotection in the newly colonized land environment. In fact, because the PSBS-dependent NPQ mechanism evolved later, it could be established only as being superimposed on the ancestral mechanism so as to ensure a sufficient level of continual protection. In the absence of NPQ, in fact, a strong decrease in fitness and fast counterselection would be expected, as shown in the case of *psbs*-less *Arabidopsis* mutants (8) and consistent with the effect of the high light treatment in the triple mutant (Fig. 5).

## Materials and Methods

**Plant Growth.** Protonemal tissue of *P. patens*, Gransden WT strain, was grown on minimum PpNO<sub>3</sub> medium (35) solidified with 0.8% Plant Agar (Duchefa Biochemie). Plants were propagated under sterile conditions on 9-cm Petri dishes overlaid with a cellophane disk (A.A. Packaging Limited), as previously described (20). Plates were placed in a growth chamber under controlled conditions: 24 °C, 16-h light/8-h dark photoperiod, and a light intensity of 40 μE·m<sup>-2</sup>·s<sup>-1</sup> (control conditions). For high light tests, 5-day-old plants were moved from control to 350 μE·m<sup>-2</sup>·s<sup>-1</sup>, maintaining temperature and photoperiod.

**NPQ Measurements.** In vivo chlorophyll fluorescence in *P. patens* was measured at room temperature with a Dual PAM-100 fluorometer (Heinz Walz GmbH, Germany), with saturating light of 6,000 μE·m<sup>-2</sup>·s<sup>-1</sup> and actinic light of 830 μE·m<sup>-2</sup>·s<sup>-1</sup>. In the case of *Arabidopsis*, plants were grown at 100 μE·m<sup>-2</sup>·s<sup>-1</sup> in an 8-h light/16-h dark photoperiod and were measured with 1,200 μE·m<sup>-2</sup>·s<sup>-1</sup> of actinic light. Before measurements, plates were dark-adapted for 40 min at room temperature. The parameters Fv/Fm and NPQ were calculated as (Fm–Fo)/Fm and (Fm–Fm′)/Fm′ (36). Antenna size was determined by the rising time of PSII fluorescence in DCMU-treated plants.

**Protoplast Transformation and KO Generation.** Genomic *P. patens* protonemal DNA was extracted (37) and used as starting template for all molecular cloning. All regions up and downstream of target coding sequences were amplified by PCR and subcloned into pGEM-T Vector (catalog no. A3600; Promega). Regions from *PSBS* (locus XM\_001778511), *LHCSR1* (locus XM\_001776900), and *LHCSR2* (locus XM\_001768019) genes were successively cloned into BZRf/BNRr, BHRf, and BNRf plasmids (kindly provided by F. Nogue, Institut National de la Recherche Agronomique, Versailles, France), respectively. *P. patens* transformation was performed as in the study by Schaefer and Zryd (24) with minor modifications. Briefly, 5- to 6-day-old protonemal tissue was collected for protoplast generation and PEG-mediated transformation. Resistant colony selection started 6 days after transformation by transferring regenerated plants on culture media supplemented with the appropriate antibiotic [50 μg·L<sup>-1</sup> G418 (Sigma-Aldrich); 30 μg·L<sup>-1</sup> Hygromycin B (Sigma-Aldrich), or 50 μg·L<sup>-1</sup> zeocin (Duchefa Biochemie)]. Resistant colonies were transferred for 10 days on nonselective media and then again in antibiotic media to isolate stable transformants. Confirmation of DNA insertion was performed by PCR assay, as detailed in Table S1.

**Thylakoid Extraction, SDS/PAGE, and Western Blotting Analyses.** Thylakoids from protonemal tissue (10- to 12-day-old plants) were prepared using an *Arabidopsis* protocol with minor modifications (20). After SDS/PAGE, proteins were transferred onto a nitrocellulose membrane (Sartorius AG) using a blot system from Biorad and were detected with specific polyclonal antibodies produced in the laboratory.

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