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PHOTOPROTECTION MECHANISMS IN THE MOSS *PHYSCOMITRELLA PATENS*: INSIGHTS ON THE PHOTOSYNTHESIS ADAPTATION DURING LAND COLONIZATION

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SUMMARY (ENGLISH)

Oxygenic photosynthesis is a crucial process for life on earth as it enables plants, algae and cyanobacteria to convert sunlight into chemical energy, generating molecular oxygen as a byproduct. Light can also be harmful and excess light intensities can drive to photosystems over-excitation, production of reactive oxygen species (ROS) and cell damage. Thus, plants and algae evolved several photoprotective processes to survive in a variable environment. The fastest one among them is called Non Photochemical Quenching (NPQ), which consists in the thermal dissipation of excess energy absorbed triggered by the generation of a ΔpH gradient across thylakoid membranes. The main and fastest component of NPQ is called "Energy quenching" or "feedback de-excitation" (qE) and it is activated in a few seconds upon an increase in light irradiation. Although widespread among oxygenic photosynthetic organisms, NPQ shows important differences in its machinery: in vascular plants as *Arabidopsis thaliana* it depends on the presence of the Lhc-like protein PSBS, while a different polypeptide, LHCSR (previously called Li818), is required in algae as the Chlorophyta *Chlamydomonas reinhardtii* and diatoms.

In this work we used the moss *Physcomitrella patens* as a new model organism for the study of photosynthesis and NPQ in particular. The choice of this moss is justified by several reasons. First of all, *Physcomitrella patens* diverged from higher plants soon after land colonization and its analysis can provide information on adaptation to terrestrial environment and evolution of NPQ process from unicellular algae to higher plants. Consistent with its peculiar position in evolution of photosynthetic organisms, in *Physcomitrella* genome both PSBS and LHCSR were identified. A further reason to study this moss is that *Physcomitrella* is able to integrate efficiently foreign DNA by homologous recombination (gene targeting), which is a unique feature among plants. Thus, it is relatively simple to obtain targeted knock-out plants with higher precision and frequency compared with seed plants, such as *Arabidopsis*.

We generated *P. patens* knock-out (KO) mutants depleted in PSBS and/or LHCSR proteins (the ones involved in vascular plants and algae NPQ, respectively) exploiting *Physcomitrella* ability of doing homologous recombination. We obtained single KO mutants depleted in PSBS (*psbs* KO) and in each of the two isoforms of LHCSR present in *Physcomitrella* genome (*lhcsr1*KO and *lhcsr2*KO), as well as double KO mutants (*psbs*

lhcsr1 KO, *psbs lhcsr2* KO and *lhcsr1 lhcsr2* KO) and the triple KO mutant, depleted in all the three proteins analyzed (*psbs lhcsr1 lhcsr2* KO).
All single and double KO mutants showed a decreased NPQ capacity compared with WT mosses, but all of them retain a residual NPQ capacity, still identifiable as qE, demonstrating that both PSBS and LHCSR play a role in *Physcomitrella* NPQ, with LHCSR1 having the largest effect. Only the triple KO depleted in all PSBS and LHCSR isoforms showed a completely abolished NPQ capacity. Its phenotype is very close to the one of *Arabidopsis* plants depleted in PSBS, again clarifying that both PSBS- and LHCSR-dependent NPQ mechanisms are indeed active in this moss.

The different KO mutants were also grown in different light and temperature conditions to evaluate the effect of the NPQ reduction. The ones having the strongest reduction in NPQ also showed the strongest photoinhibition, demonstrating that NPQ capacity is important for *Physcomitrella* photoprotection.
When grown in high light conditions for several days WT mosses showed an enhanced NPQ which was correlated with the increased expression of both PSBS and LHCSR. The modulation of PSBS or LHCSR accumulation is thus a method for modulation NPQ and provide optimal photoprotection according to the growth conditions.

We also over-expressed (OE) PSBS and LHCSR proteins in the KO lines previously obtained, and we were able to show that in *P. patens* PSBS and LHCSR are alone capable of activating a strong NPQ. Their activity appears to be largely independent, suggesting a different and autonomous activation mechanism.

The carotenoid zeaxanthin has a fundamental role in several protection mechanisms and its synthesis also enhances NPQ. We also showed that although a part of NPQ is induced also in the absence of zeaxanthin, the synthesis of this carotenoid is fundamental for the activation of a strong response in *Physcomitrella*. Zeaxanthin presence enhance similarly both PSBS and LHCSR–dependent NPQ but it is unable to induce any quenching in their absence.

In this thesis we exploited *Physcomitrella* ability of gene targeting also to express mutated form of PSBS protein. We obtained two deleted isoforms, one with a N-terminal deletion while the other truncated in the C-terminus, as these regions have conserved basic and acidic residues, respectively. PSBS function is likely driven by interaction with other proteins and these charged residues may be essential for these protein-protein interactions and thus for PSBS activity. The expression of mutated PSBS

proteins in *psbs* KO genotype complemented the effect of PSBS knocking-out, however, demonstrating that the deleted regions are not required for PSBS function.

As mentioned above, the study of *P. patens* can provide information on the adaptation to terrestrial environment. To obtain additional information we also analyzed NPQ in some Streptophyta algae (also known as Charophyta). Only the groups which diverged later with respect to plants, Coleochaetales and Charales, show the presence of a PSBS-dependent NPQ. The analysis of these algae, together with the one of *Physcomitrella patens* and literature data allows proposing an hypothesis for the evolution of NPQ. In correspondence to land colonization a new mechanism for NPQ, dependent on PSBS, evolved. This was at first in addition to the one dependent on LHCSR already present in green algae, which was subsequently lost by vascular plants.

RIASSUNTO (ITALIANO)

La fotosintesi riveste un'importanza fondamentale per la biosfera: grazie a questo processo l'energia solare è utilizzata per fissare l'anidride carbonica atmosferica in biomassa, liberando ossigeno. Piante e alghe, però, sono molto spesso esposte a condizioni d'illuminazione molto variabili. In condizioni di elevata illuminazione è fondamentale che l'energia in eccesso venga dissipata, pena la formazione di specie radicaliche dell'ossigeno (ROS) molto dannose per la cellula.
Uno dei principali sistemi di protezione dall'eccesso di luce viene attivato pochi secondi dopo una variazione di illuminazione ed è definito Non Photochemical Quenching (NPQ). L'NPQ permette di dissipare l'eccesso di stati eccitati della clorofilla come calore ed è attivato a seguito dell'acidificazione del pH lumenale. L'NPQ comprende diverse componenti, la più rapida delle quali è definita qE (da "Energy quenching" o "Feedback de-excitation"). L'NPQ è presente in tutti gli organismi fotosintetici anche se meccanismi di attivazione e proteine coinvolte sono diversi. Nelle piante vascolari (quali la pianta modello *Arabidopsis thaliana*) è noto che l'NPQ dipende da una proteina presente nelle membrane dei tilacoidi chiamata PSBS. Nelle alghe, quali diatomee o la Clorofita *Chlamydomonas reinhartii*, che pure sono in grado di attivare NPQ, PSBS è assente e l'attivazione dipende da una proteina diversa, detta LHCSR.
In questo lavoro è stato utilizzato il muschio *Physcomitrella patens* come organismo modello per lo studio della regolazione della fotosintesi. Questa scelta è dettata da molteplici ragioni. Le briofite presentano un'interessante posizione evolutiva, dal momento che si sono separate dalle piante vascolari poco dopo la colonizzazione delle terre emerse: il loro studio può quindi fornire informazioni su quelli che sono i primi adattamenti degli organismi fotosintetici alla vita terrestre. Per quanto riguarda i meccanismi di fotoprotezione, *Physcomitrella patens* ha nel proprio genoma sia PSBS che LHCSR, le proteine indispensabili per l'NPQ nelle piante vascolari e nelle alghe. *Physcomitrella*, inoltre, presenta la capacità unica tra le piante analizzate fino ad ora di fare ricombinazione omologa, facilitando così la possibilità di ottenere mutanti specifici per le proteine di interesse.

Nel particolare, in questo lavoro abbiamo ottenuto dei mutanti specifici per PSBS e LHCSR, le proteine caratteristiche dell'NPQ di piante ed alghe rispettivamente. Sfruttando la capacità di ricombinazione omologa di *Physcomitrella*, sono stati generati mutanti knock-out (KO) per PSBS (*psbs* KO) e le due isoforme di LHCSR (LHCSR1 e LHCSR2; *lhcsr1* KO e *lhcsr2* KO) presenti nel genoma del muschio. Queste mutazioni sono

poi stati combinate in doppi KO (*psbs lhcsr1* KO, *psbs lhcsr2* KO and *lhcsr1 lhcsr2* KO) ed in un triplo KO (*psbs lhcsr1 lhcsr2* KO), genotipo in cui è stato eliminato il gene di tutte tre le proteine in analisi. La risposta di NPQ nelle varie linee di singoli e doppi KO ha dimostrato come tutti questi mutanti diminuiscano la loro capacità di indurre NPQ rispetto a muschi WT. Ciò nonostante, tutte queste linee sono comunque in grado di attivare una risposta qE, dimostrando che queste proteine non solo sono presenti nel genoma ma sono entrambe attive nell'NPQ. Inoltre, l'isoforma LHCSR1 risulta essere la maggiore responsabile dell'NPQ in questa specie. Al contrario, mutanti privi dei tre polipeptidi (triplo KO) non sono in grado di indurre alcuna risposta qE, con un fenotipo simile ai mutanti di *Arabidopsis* privi di PSBS. In *Arabidopsis* PSBS è l'unica proteina coinvolta nel qE, dal momento che le piante vascolari non possiedono geni per LHCSR, confermando ulteriormente che in *Physcomitrella* sia PSBS che LHCSR sono attive nell'NPQ.

Gli effetti di una diminuita capacità di indurre NPQ sulla fotoprotezione di *P. patens* sono stati ulteriormente approfonditi analizzando la capacità di acclimatazione ad alta luce o basse temperature nel muschio WT e nelle varie linee di mutanti KO ottenute. I mutanti che già in condizioni di controllo mostravano la maggiore diminuzione di NPQ in queste condizioni di crescita mostrano anche la maggiore fotoinibizione, dimostrando l'importanza dell'NPQ per la fotoprotezione. Nei muschi WT, l'acclimatazione ad alta luce o basse temperature, infatti, è accompagnata da un aumento della capacità di attivare la risposta di NPQ che correla con un aumento dell'espressione sia di PSBS che LHCSR. Questi dati dimostrano che *Physcomitrella* è in grado di modulare la sua capacità di indurre NPQ modificando l'espressione delle proteine chiave di questo processo, PSBS e LHCSR, per ottenere una fotoprotezione adeguata alle condizioni di crescita in cui si trova.

In aggiunta a mutanti KO per PSBS e LHCSR, durante questo lavoro sono state generate anche delle linee che over-esprimono (OE) queste proteine. Questi mutanti hanno permesso di dimostrare che sia PSBS che LHCSR sono in grado di attivare una risposta NPQ in *Physcomitrella* in assenza dell'altra proteina. Questo risultato dimostra che PSBS e LHCSR sono attive in modo indipendente l'una dall'altra, ed hanno quindi verosimilmente dei meccanismi di attivazione differenti ed autonomi.

Anche i carotenoidi, e la zeaxantina in particolare, hanno un ruolo fondamentale nella protezione delle cellule dagli stress ossidativi dal momento che sono coinvolti in diversi meccanismi di fotoprotezione. Tra gli altri, la zeaxantina è in grado di amplificare la

risposta NPQ in piante quali *Arabidopsis*. In *Physcomitrella* abbiamo ottenuto lo stesso risultato, dimostrando che la presenza di questo carotenoide è fondamentale per una completa attivazione di NPQ sia PSBS che LHCSR dipendente. Se queste due proteine non sono presenti, invece, il solo accumulo di zeaxantina non è in grado di attivare alcuna risposta di fotoprotezione rapida qE.

Durante questo progetto di Dottorato è stata sfruttata la capacità di ricombinazione omologa di *Physcomitrella* anche per l'espressione di isoforme mutate di PSBS. Nel particolare, sono state ottenute le sequenze codificanti per PSBS con una delezione all'N- o al C- terminale della proteina matura. Si tratta di regioni conservate in PSBS di diverse specie di piante, che presentano rispettivamente carica positiva e negativa, e potrebbero quindi essere coinvolte in interazioni proteina-proteina che sembrano essere necessarie per il meccanismo di induzione NPQ PSBS-dipendente. L'espressione di queste isoforme in muschi *psbs* KO, però, ha portato alla complementazione del fenotipo in entrambi i casi, dimostrando che le regioni delete non sono fondamentali per l'attività di PSBS.

Nell'ultima parte del lavoro, inoltre, abbiamo focalizzato l'attenzione sull'evoluzione del meccanismo di NPQ da alghe a piante. Come accennato in precedenza, *Physcomitrella* presenta una posizione evolutiva chiave per ottenere informazioni sugli adattamenti necessari alla colonizzazione delle terre emerse ed abbiamo esteso l'analisi anche ad alghe della linea Streptofita (alghe Carofita) per ottenere maggiori informazioni sull'evoluzione dell'NPQ. I dati ottenuti hanno dimostrato che nelle specie evolutivamente più vicine alle piante terrestri (Coleochatales e Charales, identificati come i probabili "sister group" delle piante terrestri) PSBS è presente. Queste analisi, unitamente a quanto riportato in precedenza per *P. patens* e a dati di letteratura, permettono di formulare un'ipotesi sull'evoluzione dell'NPQ da alghe a piante: in corrispondenza della colonizzazione delle terre emerse è stato evoluto un nuovo meccanismo di fotoprotezione (PSBS dipendente), che dapprima si è affiancato, poi sostituito completamente a quello LHCSR-dipendente tipico delle alghe.

CHAPTER 1

INTRODUCTION

1. OXYGENIC PHOTOSYNTHESIS

Life on earth depends on solar energy and oxygenic photosynthesis enables plants, algae and cyanobacteria to convert light into chemical energy, generating molecular oxygen as a secondary product. The whole process can be divided into a light and a dark phase. This classification is based on the dependence on solar energy to occur: while the first is strictly dependent on light, the latter occurs also in the dark, provided that ATP and NADPH are available.

In photosynthetic eukaryotes, both light and dark reactions take place in a specialized organelle called chloroplast (Figure 1). The chloroplast is limited by two membranes (called together *envelope*): the outer one is highly permeable, while the inner membrane contains specific transporters which control the flux of metabolites with the cytoplasm. The envelope separates a compartment called *stroma*, which contains all enzymes catalyzing the dark reactions as well as the plastidial DNA, RNA and ribosomes.

A third membrane system, called *thylakoids*, is found in the stroma: thylakoid membranes form a physically continuous three-dimensional network which encloses a aqueous space, the thylakoid *lumen*. Thylakoids are organized in stacked regions called grana which are connected by the stroma lamellae. All protein complexes catalyzing the light reactions of photosynthesis are located in the thylakoid membranes.

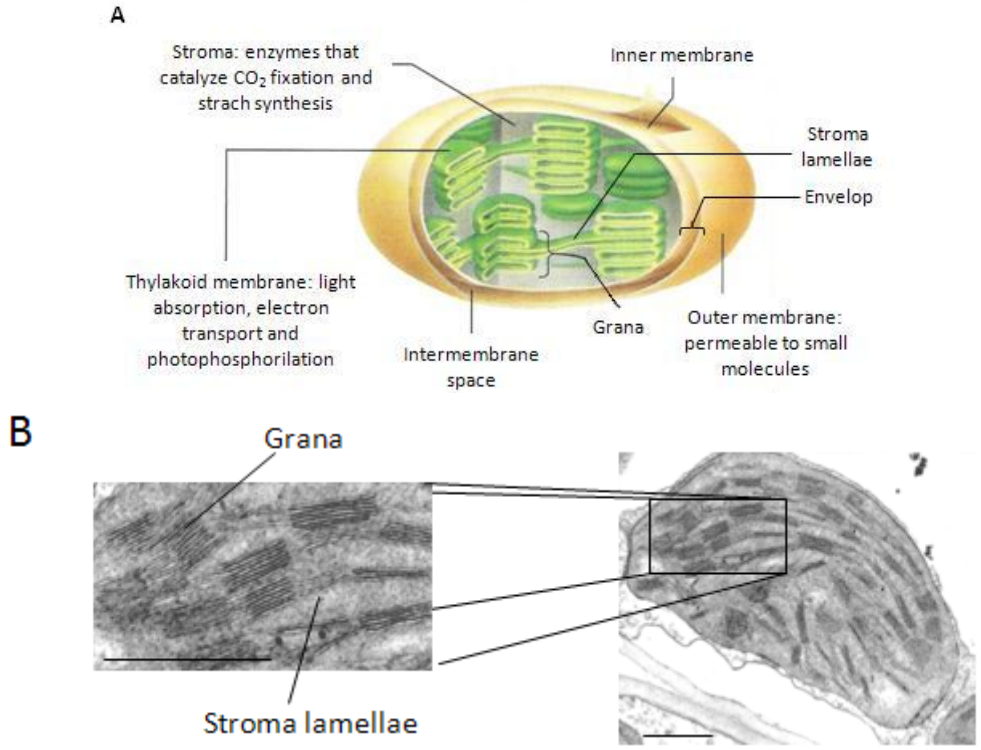

Figure 1. The chloroplast. A) Schematization of chloroplast structure, image adapted from Lodish *et al.*, "Molecular cell biology"; B) Micrograph of *Triticum durum* chloroplast. Bar: 1µm.

1.1. THE LIGHT PHASE

The light phase starts with the absorption of light by photosynthetic pigments, chlorophylls and carotenoids, bound to the antenna complexes of the photosystems (see also paragraph 2). The energy harvested is then transferred to the reaction center where it is exploited for a charge separation. A set of electron transfer processes then leads to the formation of a proton gradient across the thylakoid membrane and finally to the generation of free energy and reducing power, in the form of ATP and NADPH + H^+. Water is the primary electron donor during this process and O_2 is formed as a by-product upon its oxidation. The light reactions can be summarized with the following equations:

$2\ NADP^+ + 2\ H_2O + light \rightarrow 2\ NADPH + O_2 + 2H^+$

$ADP + P_i + energy \rightarrow ATP$

Four major protein complexes localized in the thylakoids membrane (Photosystem II, cytochrome *b_6f* complex, Photosystem I and ATP synthase, Figure 2) catalyze the processes of light harvesting, electron transport and photophosphorylation, leading to the conversion of light energy to chemical free energy (ATP and NADPH).

Taking account of the efficiency of the system, the energy required for the generation of NADPH cannot be provided by only one photon in the visible range of light and two photosystems (PS) called PSI and PSII act in series (figure 2), as described in the Z-scheme of Bendall and Hill (Hill & Bendall, 1960).

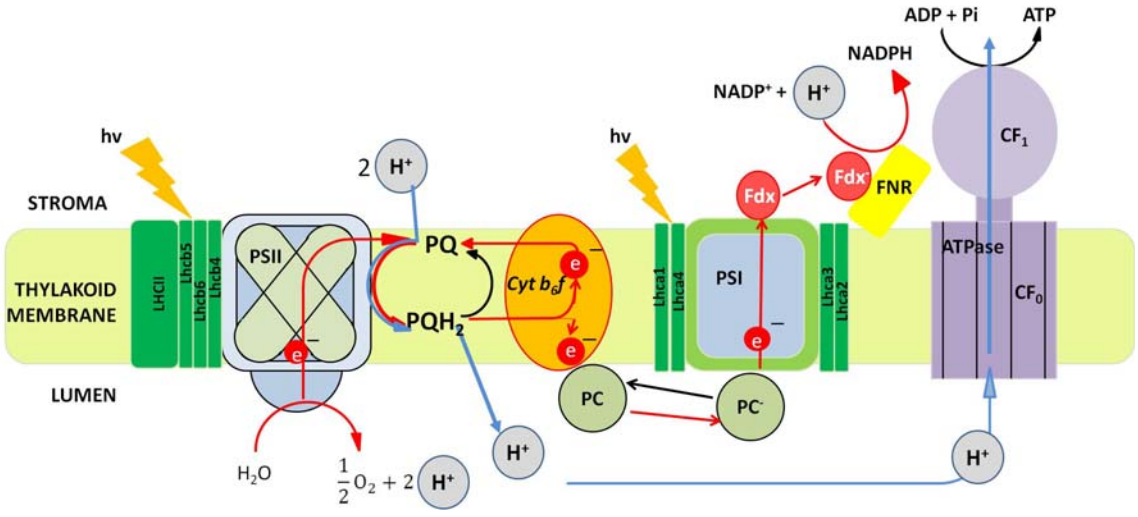

Figure 2. The light phase of photosynthesis. A schematic organization of the major protein complexes in the thylakoid membranes and of electron transport chain is shown. PSII, photosystem II; Cyt b_6f, Cytochrome b_6f, PSI, photosystem I; ATPase, ATP synthase (CF_0, CF_1, subunits of ATPase); PQ/PQH_2, plastoquinone/plastoquinol; PC, plastocyanin; Fdx, ferredoxin; FNR, Ferredoxin $NADP^+$ reductase; hv, light energy.

Besides converting excitation energy into reducing power in the form of NADPH, the electron transfer reactions of PSII and PSI contribute to the formation of an electrochemical potential across the thylakoid membrane. In fact, the primary charge separation creates an electrical potential and a difference in proton concentration between the stromal and the lumenal side of the membrane is built up by several processes (figure 2):

- During the oxidation of water, 4 protons are released on the lumenal side of the thylakoid membrane for each O_2 molecule produced.
- Protons are translocated through the thylakoid membrane during the so-called Q-cycle, which describes the reduction and the oxidation of plastoquinone/plastoquinol by the Cyt b_6f complex. The plastoquinone is reduced to plastoquinol on the stromal side of the thylakoid membrane whereas the oxidation takes place on the lumenal side, thus resulting in a net proton transport. Overall, the Q-cycle oxidizes two plastoquinols, reduces one plastoquinone, and translocates 3 H^+ for every 2 electrons transported to PSI.
- Protons are used on the stromal side of the thylakoid membrane for the reduction of each $NADP^+$ by the FNR.

The proton gradient generated is utilized by ATP synthase to produce ATP from ADP and P_i, in a process called photophosphorylation. It is worth underlining that the proton accumulation in the lumen also has a feedback control on light harvesting capacity, as detailed discuss in paragraph 3 of the introduction.

1.2. THE DARK PHASE

The dark phase of photosynthesis include a series of reactions, called Calvin-Benson cycle (Benson & Calvin, 1950), where atmospheric CO_2 is reduced to carbohydrates, using the chemical free energy (ATP and NADPH) produced during the light reactions.
The Calvin cycle consists in the net synthesis of one glyceraldehyde 3-phospate (GAP) from three CO_2 molecules and in the regeneration of Ribulose 1,5-bisphospate (RuBP) to preserve the cyclic character of the process. Nine ATP and six NADPH are consumed for the production of one GAP.

2. THE LIGHT ABSORBING COMPLEXES: PHOTOSYSTEM II AND PHOTOSYSTEM I

As mentioned, protein complexes responsible for light conversion into chemical energy, photosystems II and I (PSII and PSI), are localized in the thylakoid membranes, but their distribution is not homogeneous: in fact, PSII complex is located in the grana, while PSI in the stroma lamellae.

Although they are structurally different, in both photosystems two moieties can be identified: a core complex, responsible for charge separation, and an antenna complex, responsible for increasing the light harvesting capacity and transferring the absorbed energy to the reaction center. Reaction centers structure is widely conserved during evolution with only small differences from cyanobacteria to seed plants. Core complexes are composed by the products of the genes called *Psa* and *Psb* for PSI and PSII, respectively, encoded either by nuclear or chloroplast genomes. On the contrary, antenna systems are more variable among photosynthetic organisms (Green & Durnford, 1996; Nelson & Ben Shem, 2005; Alboresi *et al.*, 2008). In green algae and land plants, the antenna system is composed by polypeptides members of the Light Harvesting Complexes (Lhc) family: these proteins have three transmembrane helices and bind Chl *a*, Chl *b* and xanthophylls (Green & Durnford, 1996). In *Arabidopsis thaliana*, six different isoforms were identified associated to PSI (Lhca1-6) and eight to PSII (Lhcb1-8) (Jansson, 1999; Klimmek *et al.*, 2006). An additional ninth Lhcb (Lhcb9) was recently identified in a moss (Alboresi *et al.*, 2008).

2.1. PHOTOSYSTEM II: REACTION CENTER, ANTENNA COMPLEXES AND SUPRAMOLECULAR ORGANIZATION

PSII catalyses the electron transfer from water to plastoquinone (PQ) (Figure 3A). The complex is composed by different subunits encoded by both nuclear and chloroplast genomes (*Psb* genes). It contains four large membrane intrinsic subunits (PsbA-D), three membrane extrinsic subunits (PsbO-Q) and a large number of small subunits, most of which with one transmembrane helix (in crystal structures 14 transmembranes helices attributable to small subunits are observed, see also figure 3B) (Dekker & Boekema, 2005).

The core complex contains the reaction center of PSII where the primary photochemistry takes place. Three subunits, D1 (PsbA), D2 (PsbD) and Cyt b_{559}, coordinate the electron

transport cofactors P680 (the reaction center), pheophytin, Q_A and Q_B. CP43 (PsbC) and CP47 (PsbB), instead, bind respectively 14 and 16 Chl *a* molecules and compose the inner antenna system (Ferreira *et al.*, 2004). On the lumenal side of PSII three extrinsic proteins compose the oxygen evolving complex (OEC). They are responsible for the water oxidation and the generation of protons, electrons and molecular oxygen.

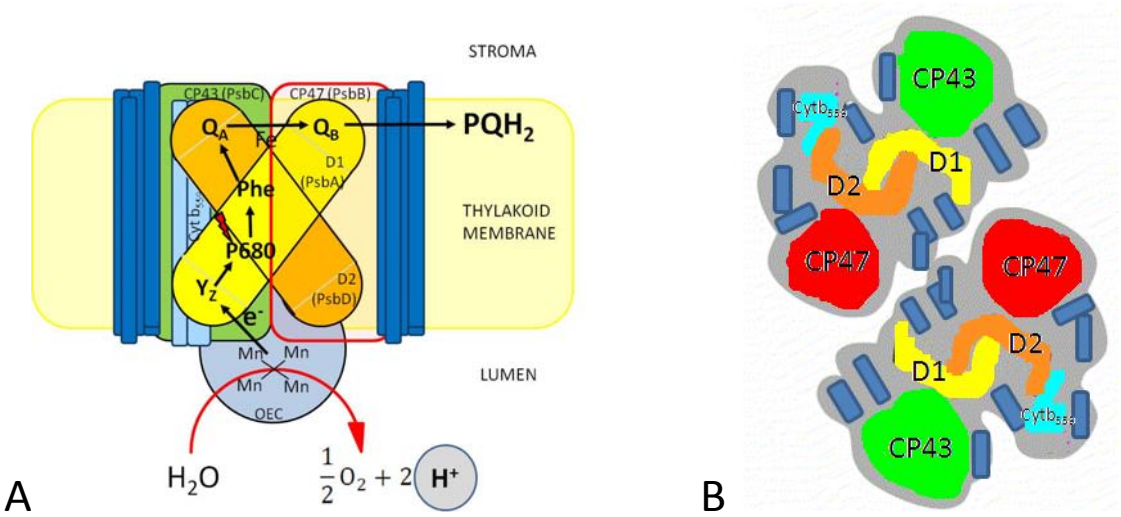

Figure 3. Schematic view of PSII core complex. Inner antennae CP43 (green) and CP47 (red), D1 (yellow), D2 (orange) and cyt b_{559} (cyan) are shown; blue bars indicate alfa-helices of the small subunits. A) Cartoon of PSII core monomer with Oxygen evolving complex (OEC) at the lumenal side. Electrons transport from water to plastoquinone is also shown. B) View from the lumen of PSII core dimer. Image B is adapted from (Hankamer *et al.*, 2001)

The major component of PSII antenna complex is called LHCII, also known as "major antennae". This complex is composed of heterotrimers of Lhcb1, 2 and 3. The three polypeptides, however, are not equimolar, with Lhcb1 found in larger amounts (Caffarri *et al.*, 2004; Dekker & Boekema, 2005). The trimeric LHCII is the only Lhc complex whose structure has been solved at high resolution (Liu *et al.*, 2004). This structure showed the presence of 14 chlorophyll (8 Chl *a* and 6 Chl *b*) and 4 carotenoid molecules per monomer (figure 4).

Lhcb4, Lhcb5 and Lhcb6 are instead classified as minor antennae, and are also known as CP29, CP26 and CP24, respectively. They are found as monomers and bind from 8 to 10 Chls and 2-3 carotenoid molecules per polypeptide (Bassi *et al.*, 1993; Ballottari *et al.*, 2010).

Additional Lhcb polypeptides (Lhcb7 and 8) have been identified in plants, but they are accumulated at lower levels with respect to Lhcb1-6 proteins (Klimmek *et al.*, 2006).

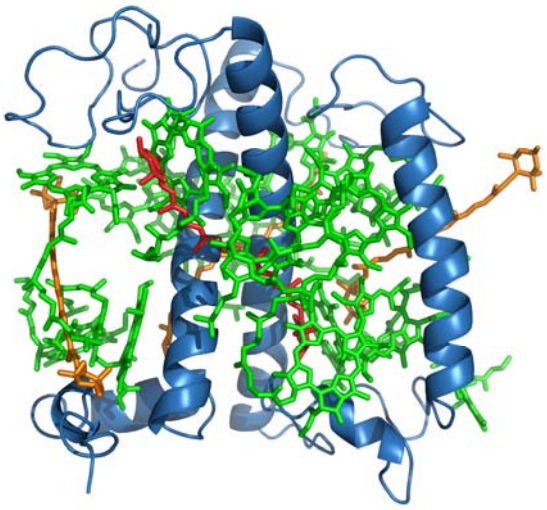

Figure 4. Tridimensional structure of an LHCII monomer. Protein backbone is shown in blue, chlorophyll ligands in green while carotenoids in orange (Adapted from (Liu *et al.*, 2004))

Photosystem II core complex forms a supramolecular organization together with its antenna. PSII core dimers are associated with a variable number of the peripheral antenna proteins to form the PSII-LHCII supercomplexes (Figure 5, (Nield *et al.*, 2000b; Dekker & Boekema, 2005)). In particular, the so-called C_2S_2 supercomplex contains a dimeric PSII core (C_2), two LHCII trimer (S_2, S stands for strongly bound) and two copies of both CP29 and CP26 (Figure 5). Larger complexes binding additional antenna are also commonly observed, as $C_2S_2M_2$ which also bind the minor antenna CP24 and one additional LHCII trimer, called M for moderately bound (Dekker & Boekema, 2005).

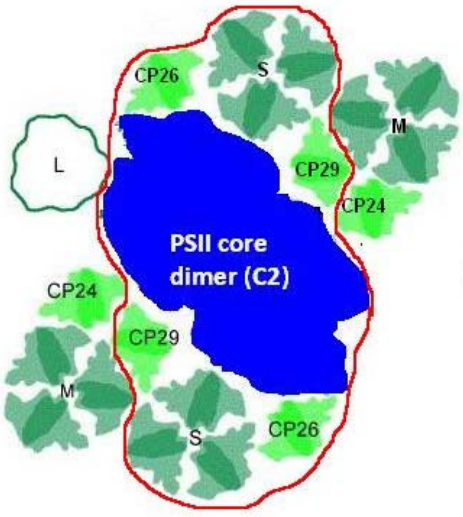

Figure 5. Supramolecular organization of $C_2S_2M_2$ supercomplexes based on electron microscopy of PSII-LHCII supercomplexes of spinach. In blue is schematized dimeric PSII core complex. Minor antennae (CP24, 26 and 29) as well as LHCII trimers (S, M) are also indicated. C_2S_2 is surrounded with a red line. In addition to S and M LHCII trimers, also additional LHCII trimers called L (L stands for loosely bound) can bind to the supercomplex. Image modified from (Dekker & Boekema, 2005)

2.2. PHOTOSYSTEM I: REACTION CENTER, ANTENNA COMPLEXES AND SUPRAMOLECULAR ORGANIZATION

The pigment-binding protein complex Photosystem I (Figure 6) is a light dependent plastocyanin-ferredoxin oxidoreductase. In higher plants it consists of at least 18 polypeptides and it binds around 180 chlorophylls and 35 carotenoid molecules (Ben Shem *et al.*, 2003).

The PSI core in higher plants is composed by 14 polypeptides (Jensen *et al.*, 2007). Among these, 10 are conserved and are also found in bacterial PSI (PsaA-F, PsaI, J, K, L) while 4 are specific of eukaryotes (PsaG, H, N, O) (Dekker & Boekema, 2005; Jensen *et al.*, 2007).

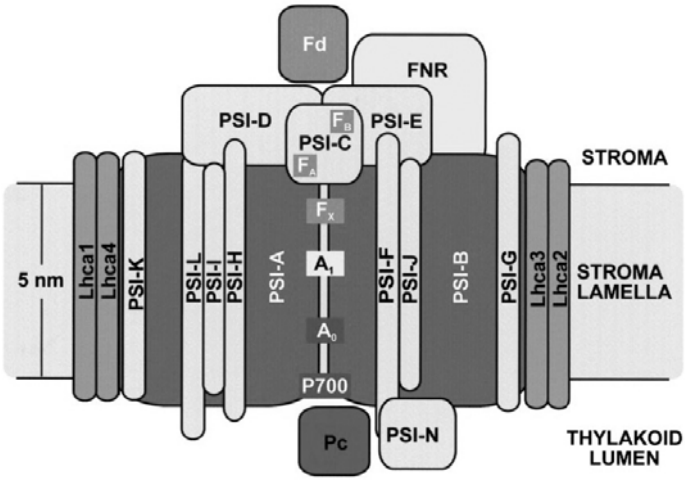

Figure 6. Schematic model of Photosystem I from plants. Image modified from (Scheller *et al.*, 2001)

Different from PSII, PSI in higher plants is a monomer and its antenna LHCI is stably associated to the core complex (Ballottari *et al.*, 2004; Morosinotto *et al.*, 2005). Figure 7 shows PSI-LHCI supercomplexes, where the LHCI subunits (Lhca1, 2, 3 and 4) bind in one cluster at one side of PSI core complex (Ben Shem *et al.*, 2003). Also in the case of PSI additional Lhca genes have been identified (Lhca 5 and 6) but they are expressed at low level (Klimmek *et al.*, 2006). Interestingly, one of them, Lhca6, has been shown to play a key role in formation of complexes with NADH dehydrogenase, a complex which mediates cyclic electron flow around PSI (Peng *et al.*, 2009). In addition to Lhca polypeptides, also LHCII can associate to PSI, in a process called state transition, probably interacting with PsaA, H, L, K, thus on the opposite side of PSI with respect to Lhca polypeptides (Figure 7; (Kouril *et al.*, 2005; Amunts *et al.*, 2007)).

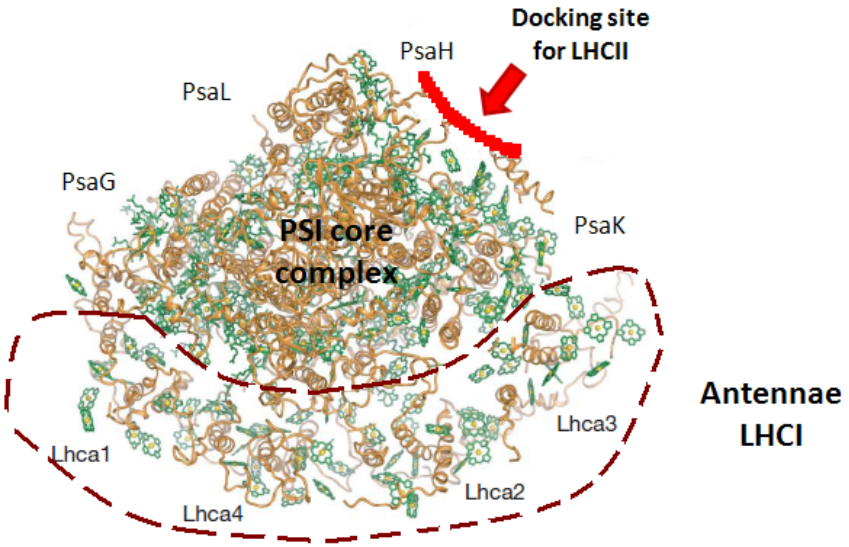

Figure 7. PSI-LHCI complex, with binding of LHCII to PSI-LHCI complex. Image modified from (Amunts *et al.*, 2007)

3. REGULATION OF LIGHT HARVESTING AND PHOTOPROTECTION

Photosynthetic organisms depend on sunlight for the energy supporting their life, but when incident light exceeds plants photosynthetic capacity it becomes a dangerous source of oxidative stress ((Barber & Andersson, 1992; Li *et al.*, 2009); see figure 8). In addition to intense illumination, several abiotic stress, such as heat, cold, drought and nutrient deficiency can lead to the over-reduction of photosynthetic electron transport chain and to the saturation of photosynthesis. As example, in low temperatures the thylakoids membrane is more rigid and the re-oxidation of the membrane soluble plastoquinol becomes a limiting factor, reducing electron transport efficiency. Furthermore cell metabolism and downstream carbon dioxide fixation reactions are also slower in low temperatures and in these conditions even a relatively weak light can exceed the photosynthetic capacity. In these conditions singlet Chl excited molecules (^{1}Chl*) are not all exploited for photochemical reactions and can convert into Chl triplets (^{3}Chl*) by intersystem crossing. Chl triplet molecules are not harmful themselves, but are stable enough to react with molecular oxygen, which normally exists as a triplet, generating singlet oxygen ($^1O_2$) and other reactive oxygen species (ROS). These molecules are instead highly dangerous since they can oxidize and degrade chloroplast components, like proteins, lipids, and pigments, leading to photo-oxidative damages and eventually to cell death (Niyogi, 1999; Havaux & Niyogi, 1999; Niyogi, 2000; Li *et al.*, 2009).

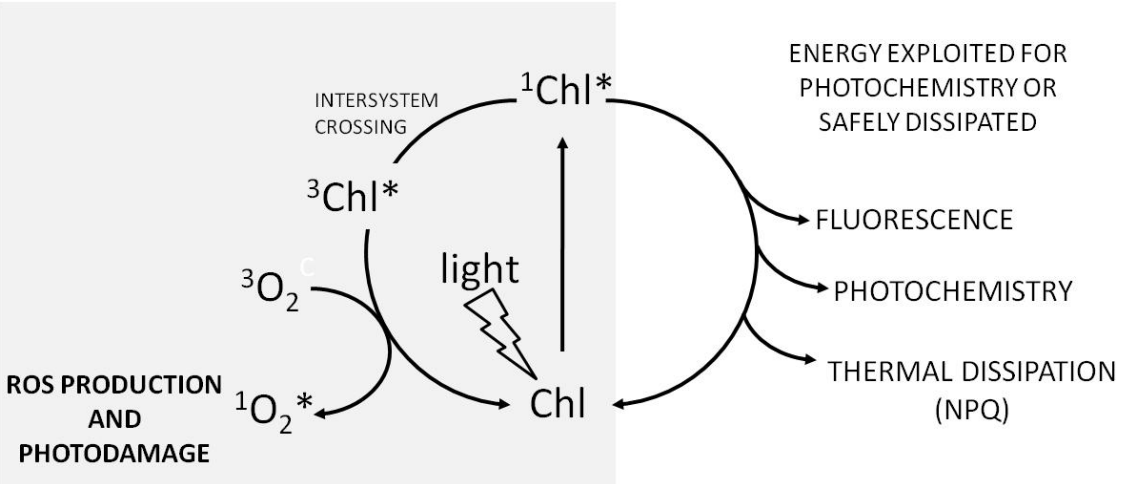

Figure 8. Pathways of ^{1}Chl* de-excitation and production of ROS. Formation of ^{3}Chl* through intersystem crossing from singlet Chl is basically an unregulated, constitutive process: the yields of ^{3}Chl* vary in proportion to the average lifetime of ^{1}Chl* in the antenna. The absorption of excess photons in strong light conditions causes accumulation of excitation energy in the antennae, as photochemical reaction and thermal dissipation processes are not able to deal with all the energy absorbed, thereby increasing the lifetime of ^{1}Chl* and the yield of ^{3}Chl*production. Chl triplet then can interact with O_2 to form singlet oxygen and ROS leading to damages in cells. NPQ: Non Photochemical quenching, see below for a description of the process. Image adapted from (Muller *et al.*, 2001)

Photosynthetic organisms evolved multiple photoprotection mechanisms to avoid damage from excess illumination. Some of them are constitutively present, such as carotenoids bound to photosystems, which are capable of efficiently scavenging chlorophyll triplets states and singlet oxygen species. Others are instead activated by strong illumination. Light irradiation can change very rapidly (e. g. as a consequence of passing clouds or of the leaves movement in a tree canopy), as well as with slow seasonal changes: plants, thus, need to be able to respond to these variations with the same speed.

A possible method to distinguish different protection mechanisms is based on their activation timescales. In particular, in a few seconds after a change in light irradiation, plants are unable to regulate their light harvesting efficiency and instead they activate quenching mechanisms that safely dissipates energy in excess as heat. On a longer timescale, on the contrary, cells can modulate their genes expression and proteins content in order to adapt their metabolism to the new environmental conditions (Niyogi, 1999; Eberhard *et al.*, 2008; Li *et al.*, 2009).

3.1. SHORT TERM REGULATION OF PHOTOSYNTHETIC APPARATUS

Pigment molecules cannot be fast synthesized and degraded and doing this would be energetically very costly. Thus, light absorption is not modifiable in a short lapse of time. On the contrary, plants evolved the ability to modulate the use efficiency of the harvested energy by regulation of the fraction of absorbed energy which is de-excited thermally (see figure 8 above). In excess light condition up to 75% of absorbed photons can be eliminated by thermal dissipation, throughout the de-excitation of ^{1}Chl*. This process is called Non Photochemical Quenching (NPQ) since it is measured by quantifying the light induced quenching of Chl fluorescence (Demmig-Adams *et al.*, 1996; Niyogi, 1999).

NPQ is a complex phenomenon and it involves several components: the fastest one, which is induced and relaxes in seconds, is known as qE (Energy-dependent quenching; see section 3.2), while a second slower component is called qI (as photoinhibitory quenching; see paragraph 3.3), and instead it relaxes in hours timescale (Figure 9) (Niyogi, 2000; Muller *et al.*, 2001; Szabo *et al.*, 2005). In some cases also a third component called qT has been proposed and attributed to the activation of state transition (see section 3.3) (Wollman, 2001; Szabo *et al.*, 2005; Eberhard *et al.*, 2008). NPQ was shown to be a key mechanism for plants survival as demonstrated by the fact

that plants unable to activate NPQ show reduced fitness in a natural environment (Kulheim *et al.*, 2002).

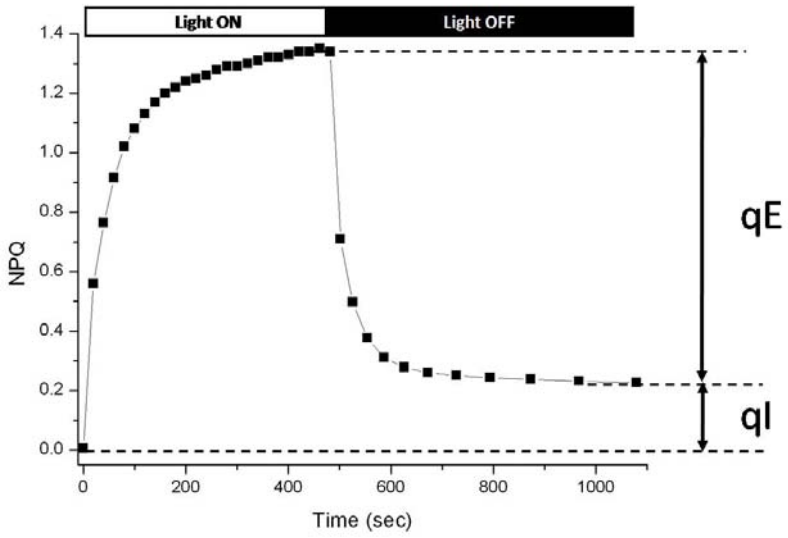

Figure 9. Induction and relaxation kinetic of NPQ in *Arabidopsis thaliana*. Starting from dark adapted leaves, NPQ is induced upon exposure of the sample to light (Light ON, white bar). The process then relaxes in the dark (Light OFF, black bar). qE and qI components are graphically identified as the quenching which relaxes in a few minutes of dark and the sustained quenching, respectively. Usually qE represents the majority of NPQ, as shown in this kinetic. See also section 5.2 for a detailed description of NPQ measurement.

Within a few minutes upon an increase in light irradiation there is also a modification in carotenoid composition: zeaxanthin is synthesized from pre-existing violaxanthin by VDE enzyme in the so-called xanthophyll cycle. Zeaxanthin is involved in many protection mechanisms inside the chloroplast, from enhancing qE capacity to the scavenging of ROS in the thylakoid membranes (Arnoux *et al.*, 2009; Jahns *et al.*, 2009) (see paragraph 3.4).

3.2. THE FASTEST COMPONENT OF NPQ: qE

The fastest component of NPQ, called qE (Energy-dependent quenching), is activated in a few seconds upon changes in illumination. The signal triggering its activation is the acidification of lumenal pH. In fact, lumenal pH decreases when the generation of chemical energy by the electron chain exceeds the capacity of assimilatory reactions, as CO_2 fixation. This allows a fine control of the fate of energy absorbed: qE is fast activated when the energy harvested exceed cell photosynthetic capacity, but it is also rapidly relaxed when light intensities decrease to exploit all energy available for CO_2 fixation in dim light conditions (Figure 9).

Since qE is controlled primarily by the amplitude of ΔpH across the thylakoids it is also known as “pH-dependent quenching” or “feedback de-excitation” (Niyogi, 2000; Muller *et al.*, 2001; Szabo *et al.*, 2005). qE is associated with quenching of Chl fluorescence *in vivo* and also to a change in absorbance at 535 nm (ΔA_{535}), which is probably due to both zeaxanthin accumulation and to a conformational change in the thylakoid membranes (Muller *et al.*, 2001).

All photosynthetic eukaryotes are able to induce a qE response although its intensity may be highly different. Moreover, its activation relies on different proteins in algae and plants. In vascular plants it depends on the presence of PSBS ((Li *et al.*, 2000) see section 3.2.1), while in algae from LHCSR polypeptide ((Peers *et al.*, 2009); see section 3.2.2).

3.2.1. PSBS PROTEIN AND VASCULAR PLANT qE

NPQ in *Arabidopsis thaliana* has been shown to depend on the presence of a protein called PSBS (Li *et al.*, 2000). Mutants depleted in this protein (*npq4* mutants) are unable to activate NPQ and in particular its fast qE component, while showing unaffected ability of photosynthesis and pigment composition.

PSBS is an Lhc-like protein of about 22 kDa localized in the thylakoid membranes. It has four transmembrane helices (figure 10), different from the typical Lhc polypeptides involved in light harvesting which have three. PSBS protein also present a twofold symmetry, as shown in figure 10 by the black and grey circles: the lumenal loops, the first and the third helices as well as the second and the forth are highly similar. Latter helices are also similar to the second helix of the 3-helix Lhc proteins, suggesting that PSBS may derive from the duplication of an ancestral gene encoding a two-helix protein, and the 3-helix Lhc successively evolved from a 4-helix ancestor like PSBS (Li *et al.*, 2000; Li *et al.*, 2004). According to this hypothesis, during evolution members of Lhc family involved in photoprotection appeared before those implicated in light harvesting (Li *et al.*, 2000). However, more recently other hypotheses for the evolution of LHC family members, with an independent evolution of 4- and 3- helices proteins, have also been proposed (Engelken *et al.*, 2010).

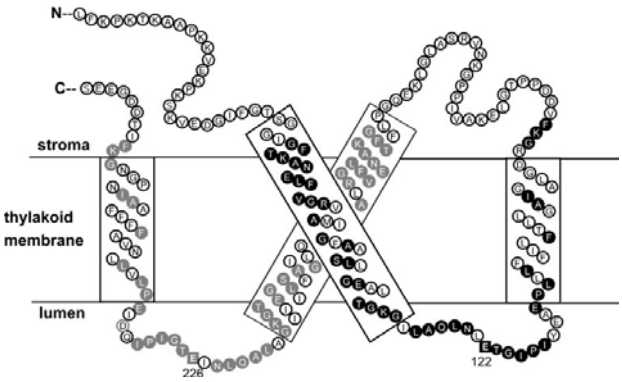

Figure 10. Topology of PSBS. Symmetrically arranged amino acid residues in the first half (black circles) and second half (grey circles) of the protein are highlighted. The two glutamates relevant for PSBS function are denoted by square symbols and numbered (E122 and E226). (Image from (Li *et al.*, 2004))

In *Arabidopsis* it has also been shown that two conserved glutamate residues located in the lumenal loops of the protein (E122 and E226, figure 10) are particularly important for PSBS activity (Li *et al.*, 2002b). Plants expressing a mutated form of PSBS protein missing these two residues (*npq4*-E122QE226Q) are unable to activate any NPQ, showing a phenotype similar to *npq4* plants where PSBS is completely absent. These results suggest that these two glutamate residues are the target of protonation upon thylakoid lumen acidification and mediate the activation of PSBS dependent qE (Li *et al.*, 2004). In addition, PSBS mutants present a semidominant loss of function phenotype: heterozygous mutants for PSBS shown about half the capacity of qE induction compared to WT plants (Li *et al.*, 2000; Li *et al.*, 2002a; Li *et al.*, 2002b; Li *et al.*, 2004).

Despite several studies, the PSBS molecular mechanism is still unclear. It was firstly proposed that PSBS was able to bind pigments, suggesting a direct role of PSBS in energy dissipation (Li *et al.*, 2000; Li *et al.*, 2002b). More recent data, however, suggest instead that PSBS is probably unable to bind pigments (Crouchman *et al.*, 2006; Bonente *et al.*, 2008a) and likely it acts as a modulator of energy dissipation efficiency in other complexes: upon protonation, it induces a conformational change in the antennae of PSII which leads to the formation of complexes with high heat dissipation efficiency (Betterle *et al.*, 2009).
Consistently, many recent works also highlight that depletion of several Lhc polypeptides like Lhcb4-6 has consequences in NPQ efficiency (Dall'Osto *et al.*, 2005; Kovacs *et al.*, 2006; De Bianchi *et al.*, 2008).

The PSBS precise role in NPQ is still unclear also because its exact localization within PSII complexes is still not defined. Based on previous data, it was initially proposed that it could be localized at the interface between the reaction centre core and the peripheral

light harvesting antenna (Li *et al.*, 2000). However, more recent studies highlight that PSBS is not a component of the purified C_2S_2 complex (Nield *et al.*, 2000a) and cannot be accommodated in the $C_2S_2M_2$ complex (Dekker & Boekema, 2005), as confirmed also by the biochemical analysis of purified $C_2S_2M_2$ supercomplexes (Caffarri *et al.*, 2009). As a consequence, PSBS must have a peripheral localization, although this has never been experimentally proven. Immunoaffinity and immunoprecipitation experiments showed that PSBS interacts with many different photosynthetic complexes (as CP29, components of LHCII, PSI, or cyt b_6f complexes), leading to the suggestion that PSBS might be mobile in the thylakoids membrane (Teardo *et al.*, 2007). Reversible dimerisation was also suggested to modulate its activity (Bergantino *et al.*, 2003), as represented also in figure 11 (Kereiche *et al.*, 2010).

To summarize, recent studies highlight that PSBS has a key role in qE in vascular plants (Li *et al.*, 2000). It is likely unable to bind pigments (Crouchman *et al.*, 2006; Bonente *et al.*, 2008a) and works in qE by sensing the lumen acidification (Li *et al.*, 2002b). Consequently, it induces a conformational change in other Lhc proteins within PSII complexes which favors energy dissipation rather than light harvesting, probably by controlling the macro-organization of PSII complexes in the grana membranes of higher plants (figure 11) (Kiss *et al.*, 2008; Betterle *et al.*, 2009; Kereiche *et al.*, 2010)

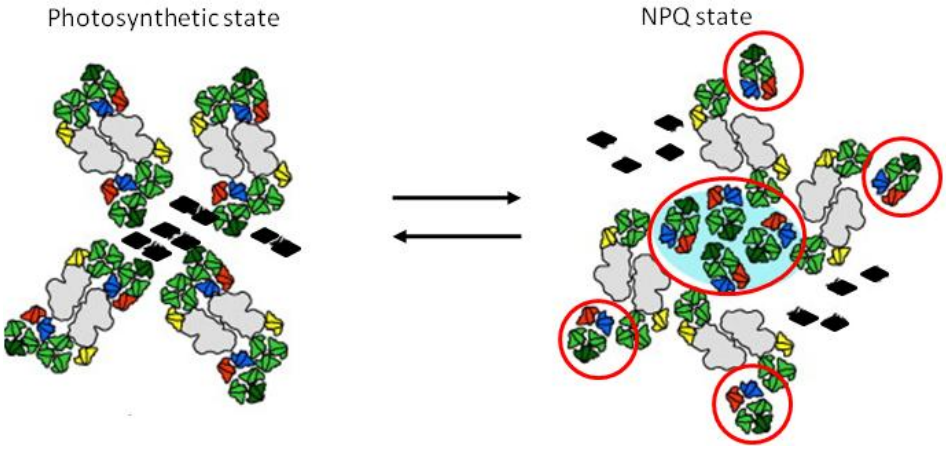

Figure 11. Scheme of PSII supercomplexes macro-organization re-modeling induced by PSBS protein. PSBS is shown as a black square. Lhcb polypeptides are shown in green (LHCII), blue (lhcb4), yellow (lhcb5) and red (lhcb6). The dissociation of "B4C" complexes composed by LHCII-M, lhcb4 and Lhcb6 which appear necessary for the establishment of NPQ (Betterle *et al.*, 2009) is highlighted by red circles. Image adapted from (Kereiche *et al.*, 2010)

3.2.2. LHCSR POLYPEPTIDE AND qE IN ALGAE

As discussed in the previous section, in *Arabidopsis* many data support the importance of PSBS for NPQ, and evidences for its role are also found for several other vascular plants (Hieber *et al.*, 2004; Bajkan *et al.*, 2010). However, the presence of PSBS protein is not ubiquitous in *Viridiplantae*. Among algae, only green algae show a conserved gene for PSBS, although the corresponding protein was never detected (Li *et al.*, 2000; Koziol *et al.*, 2007; Alboresi *et al.*, 2008; Bonente *et al.*, 2008b; Engelken *et al.*, 2010). As an example, in the green alga *Chlamydomonas reinhardtii* (a Chlorophyta) a gene for PSBS was found in the genome: the corresponding polypeptides present a 42% similarity with respect to PSBS of some higher plant, presenting also the two glutamate residues critical for its function. However, in this alga PSBS polypeptide was never detected in significant amounts, neither in stress conditions, and even the over-expression of *Cr* PSBS recombinant protein failed to produce a functional protein as PSBS polypeptide do not accumulate in the thylakoid membranes in the over-expressing lines (Bonente *et al.*, 2008b).
Despite the absence of PSBS protein, however, algae are able to induce a fast NPQ response suggesting that different proteins are likely involved in NPQ in these organisms.

More recently a new protein called LHCSR (previously known as Li818) has been identified to be responsible of *Chlamydomonas* qE (Peers *et al.*, 2009). *C. reinhardtii* mutant depleted in LHCSR (*npq4* mutant) was recently described showing a qE-deficient phenotype, as *Arabidopsis* plants depleted in PSBS (Peers *et al.*, 2009). These results demonstrated the key role of LHCSR in NPQ induction in this Chlorophyta, which was further confirmed by the analysis of *Chlamydomonas* lines over-expressing LHCSR, which showed enhanced qE capacity (Peers *et al.*, 2009). *Cr npq4* mutants also showed reduced fitness in variable light conditions, demonstrating that LHCSR is required for survival in a dynamic light environment (Peers *et al.*, 2009).

LHCSR, as PSBS, is a member of the LHC superfamily. Orthologs of LHCSR are found in many photosynthetic taxa and in particular in many different algal groups. Remarkably, however, it is missing in vascular plants genomes (Richard *et al.*, 2000; Koziol *et al.*, 2007; Alboresi *et al.*, 2008; Peers *et al.*, 2009; Engelken *et al.*, 2010). Consistent with a role in photoprotection, LHCSR transcripts accumulate in conditions which are known to induce photo-oxidative stress and it was originally identified as a light-induced transcript (called LI818) (Richard *et al.*, 2000) and thus it exhibits an expression regulation pattern

different from the other LHCs involved in light harvesting (Richard *et al.*, 2000; Peers *et al.*, 2009).

More recent data also suggest that LHCSR-dependent NPQ mechanism is likely widespread among algae, as it has been recently demonstrated to be involved in NPQ also in some diatoms (Zhu & Green, 2010; Bailleul *et al.*, 2010). Differently from *Chlamydomonas*, these organisms also completely lack of PSBS gene in their genomes (Koziol *et al.*, 2007; Engelken *et al.*, 2010).

Biochemical characterization of *Cr* LHCSR showed some similarities but also some interesting differences with respect to PSBS (Bonente *et al.*, 2011). As PSBS, LHCSR binds DCCD suggesting the presence of protonable residues and thus the ability of sensing the acidification of thylakoid lumen. Differently from PSBS, instead, LHCSR is a pigment-binding protein. *In vitro* refolding experiments showed that LCHSR stably binds Chl *a*, Chl *b*, lutein and violaxanthin/zeaxanthin. Most remarkably, spectroscopic analyses showed that LHCSR even *in vitro* shows very short fluorescence lifetimes compared to other members of Lhc family, demonstrating that LHCSR is highly efficient in dissipating energy (Bonente *et al.*, 2011).

3.3. OTHER NPQ COMPONENTS: qI AND qT

As reported in paragraph 3.1, qE is not the only component of NPQ. Compared to qE, the component called qI (photo-Inhibitory quenching) is slowly activated and also takes a longer period to relax in the dark, and thus it includes mechanisms for the sustained dissipation of excess photons. qI is normally much smaller compared to qE (see figure 9 of this introduction). As its name suggests, qI has been initially associated to photoinhibition rather than to regulation. However, it was more recently found that qI also includes mechanisms for sustained quenching induced by zeaxanthin accumulation, which is slowly reconverted to violaxanthin (Niyogi, 1999; Niyogi, 2000; Dall'Osto *et al.*, 2005; Horton & Ruban, 2005; Szabo *et al.*, 2005; Nilkens *et al.*, 2010). Although there is not yet a consensus in the literature, most likely the amount of qI attributable to photoinhibition is likely dependent on the species and measuring conditions.

In addition to qE and qI, also a third component, termed qT, has commonly been associated to NPQ. qT stands for “State transition”, which is a response to different light quality, rather than quantity. In fact, changes in the spectral composition of the incident light affect the relative excitation of the two photosystems and the overall efficiency of the electron transport chain. State transition phenomenon involves a reversible

association of the major LHCII antennae with PSII in state 1 or PSI in state 2. This process thus balance the absorption capacity of the two photosystems. (Wollman, 2001). This phenomenon was firstly identified in unicellular algae (as reviewed in (Wollman, 2001; Eberhard *et al.*, 2008)), but it is also important for plants in low light conditions (Bellafiore *et al.*, 2005). As the migration of LHCII from PSII to PSI reduced PSII antenna size, this process results in Chl fluorescence quenching, and thus it might as well influence NPQ. However, at least in plants state transition is not activated by strong illumination and the significance of this component for photoprotection is still debatable.

3.4. PHOTOPROTECTION BY CAROTENOIDS AND XANTHOPHYLL CYCLE

Carotenoids are key molecules to avoid photo-oxidative damage, as clearly shown by the fact that mutants unable to synthesize carotenoids are lethal. They protect chloroplast by different mechanisms, being involved in the de-excitation of Chl excited states as well as in the direct scavenging of ROS in the thylakoid membranes. More in detail, the deactivation of excited triplet Chl (^{3}Chl*) and singlet oxygen ($^1O_2$) in the reaction center and antenna proteins is constitutively provided by tightly bound carotenoids. In addition to this constitutive photoprotection, the light-regulated and reversible induction of a dissipative state in the antenna of PSII is also enhanced by the action of the so called xanthophyll cycle (violaxanthin cycle) (Jahns *et al.*, 2009). The violaxanthin cycle is present in all plants and green algae. In this cycle violaxanthin is de-epoxidated to antheraxanthin and then zeaxanthin, (Figure 12). Once synthesized, zeaxanthin plays a central role in photoprotection in chloroplast being involved in several protection mechanisms (Havaux & Niyogi, 1999; Jahns *et al.*, 2009). In fact, in *Arabidopsis thaliana* it contributes to the heat dissipation of excess excitation energy in the qE mechanism, by enhancing PSBS-dependent NPQ induction (Niyogi *et al.*, 1998; Holt *et al.*, 2005; Jahns *et al.*, 2009). Zeaxanthin was also suggested to be involved in slower photoprotection processes components of NPQ (Dall'Osto *et al.*, 2005; Nilkens *et al.*, 2010). Zeaxanthin bound to antenna complexes is also active in the scavenging of Chl triplets (Mozzo *et al.*, 2008) and when accumulated free in the thylakoids membrane it is an effective antioxidant for the scavenging of ROS in the membranes to avoid lipids peroxidation (Havaux & Niyogi, 1999; Demmig-Adams & Adams, III, 2002; Havaux *et al.*, 2007; Jahns *et al.*, 2009).

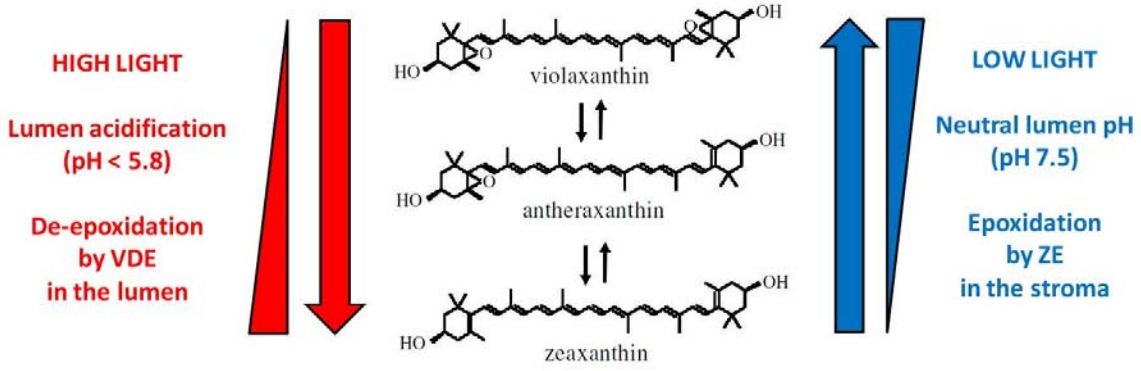

Figure 12. The violaxanthin cycle.

Reactions of violaxanthin cycle are catalyzed by the enzymes Violaxanthin de-epoxidase (VDE) and Zeaxanthin epoxidase (ZE) (figure 12).
As well as PSBS mediated photoprotection, also violaxanthin conversion to zeaxanthin is activated by the over-acidification of thylakoid lumen caused by strong light. In fact, VDE enzyme was recently shown to undergo a conformational change upon activation which depends on the lumenal pH: at neutral pH (in dim light conditions), VDE is a soluble monomeric enzyme in the thylakoid lumen, while upon acidification of the lumen it dimerizes and binds to the membrane, likely in regions of the thylakoids enriched in MGDG (Arnoux *et al.*, 2009; Jahns *et al.*, 2009; Saga *et al.*, 2010). To obtain the catalysis, moreover, the violaxanthin substrate has to be released from its binding site in antenna proteins and to diffuse in the thylakoid membranes to reach VDE (Jahns *et al.*, 2009).

As for PSBS-dependent energy dissipation, the key role of the xanthophyll cycle in photoprotection is confirmed by the analyses of mutants affected in xanthophylls conversion. *Arabidopsis* mutant *npq1* is defective in VDE and unable to produce zeaxanthin. As a consequence, it also shows increased light induced PSII damage and lipid peroxidation (Niyogi *et al.*, 1998; Havaux & Niyogi, 1999; Jahns *et al.*, 2009). On the contrary, *Arabidopsis* mutants *npq2*, defective in ZE, present a constitutive high level of zeaxanthin and shows a faster NPQ induction and a slower NPQ relaxation compared to WT plants. In addition, these mutants show an higher NPQ level at low light condition, with an observed growth reduction under limiting light (Niyogi *et al.*, 1998; Dall'Osto *et al.*, 2005; Jahns *et al.*, 2009). The effect of constitutive zeaxanthin accumulation on the growth in low light conditions underlines the importance of a precise regulation of photoprotection mechanisms, which must be rapidly activated when energy is in excess, but also fast de-activated when light is limiting to avoid unnecessary quenching.

3.5. LONG TERM RESPONSES

Plants evolved also long-term mechanisms to respond to the slow modification of their surrounding environment. This response is globally called acclimation and it involves the regulation of nuclear and chloroplast gene expression, and/or the proteolytic degradation of existing proteins, in particular antenna proteins Lhcs. One long-term response commonly observed in response to strong illumination in vascular plants is the decrease in the size of the antenna system associated with the photosystems to reduce light harvesting efficiency. Other responses are also commonly observed such as changes in PSII/PSI stoichiometry and the increase of photosynthetic electron transport and metabolic sinks to better exploit the available excitation energy.

Constitutive photoprotection processes are also activated such as the accumulation of antioxidant molecules like vitamin E or carotenoids. Furthermore, also plant morphology can be altered according to different environmental conditions (Huner *et al.*, 1998; Niyogi, 1999; Eberhard *et al.*, 2008; Li *et al.*, 2009).

4. A NEW MODEL ORGANISM FOR THE STUDY OF PHOTOSYNTHESIS: THE MOSS *PHYSCOMITRELLA PATENS*

In this work we will focus mainly on one model organism, the moss *Physcomitrella patens*.

4.1. THE BRYOPHYTES

Mosses (phylum Bryophyta), together with liverworts (phylum Marchantiophyta) and hornworts (phylum Anthocerotophyta), are known as "Bryophytes" and diverged from vascular plants (Tracheophytes) about 450 million years ago, after the colonization of terrestrial environment by their common land ancestor. Altogether, bryophytes and tracheophytes make up the Embryophytes, the land plants (Figure 13) (Waters, 2003; Bowman *et al.*, 2007).

Bryophytes are characterized by a dominant gametophytic haploid (1n) generation, with all vegetative structures (leaves, stem and rhyzoids) belonging to the 1n generation. Bryophyte gametophytes lack secondary growth and meristematic tissues, growing instead new tissue from a single apical cell. The sporophyte (2n generation) is unbranched and parasitic on the gametophyte. During evolution of vascular plants instead the gametophyte structures became progressively smaller, with present dicots (as *Arabidopsis thaliana*, see figure 13) being characterized by the dominant sporophytic (2n) generation (Glime, 2007). In addition, differently from vascular plants, bryophytes still need water for fertilization and sperm cells has to swim to reach the female reproductive structure (Glime, 2007).

Bryophytes are small organisms, from a few millimeters to about 1 meter size, and thus are forced to be simpler compared to vascular plants, where a great variety of cell types have a specialized function. One major difference is that bryophytes lack lignin and without the mechanical support from this molecule their size is limited. They also lack "true" leaves and roots: their leaves (better called "phyllids") are usually only one cell thick, so much simpler compared to vascular plants ones, and they are sustained by rhyzoids instead of roots. A further consequence of the lack of lignin is that Bryophytes do not present a vascular system, although many bryophytes possess "hydroids" and "leptoids" with the same function of xylem and floem, respectively. As a consequence of

the absence of roots, of tracheid conducting systems and of stomata in their body, Bryophytes are not able to control their state of hydration internally. Thus, they are poikilohydric organisms since their hydration state depends on the moist of the surrounding environment. However, many mosses are also desiccation-tolerant: this capacity makes them able to survive to unfavorable dry conditions (Glime, 2007).

Despite their small size and vascular limitations, bryophytes, and mosses in particular, can occupy large surface areas on rocks, soil and tree trunks, and thus physiological and biochemical adaptations abound to survive in those different habitats (Glime, 2007).

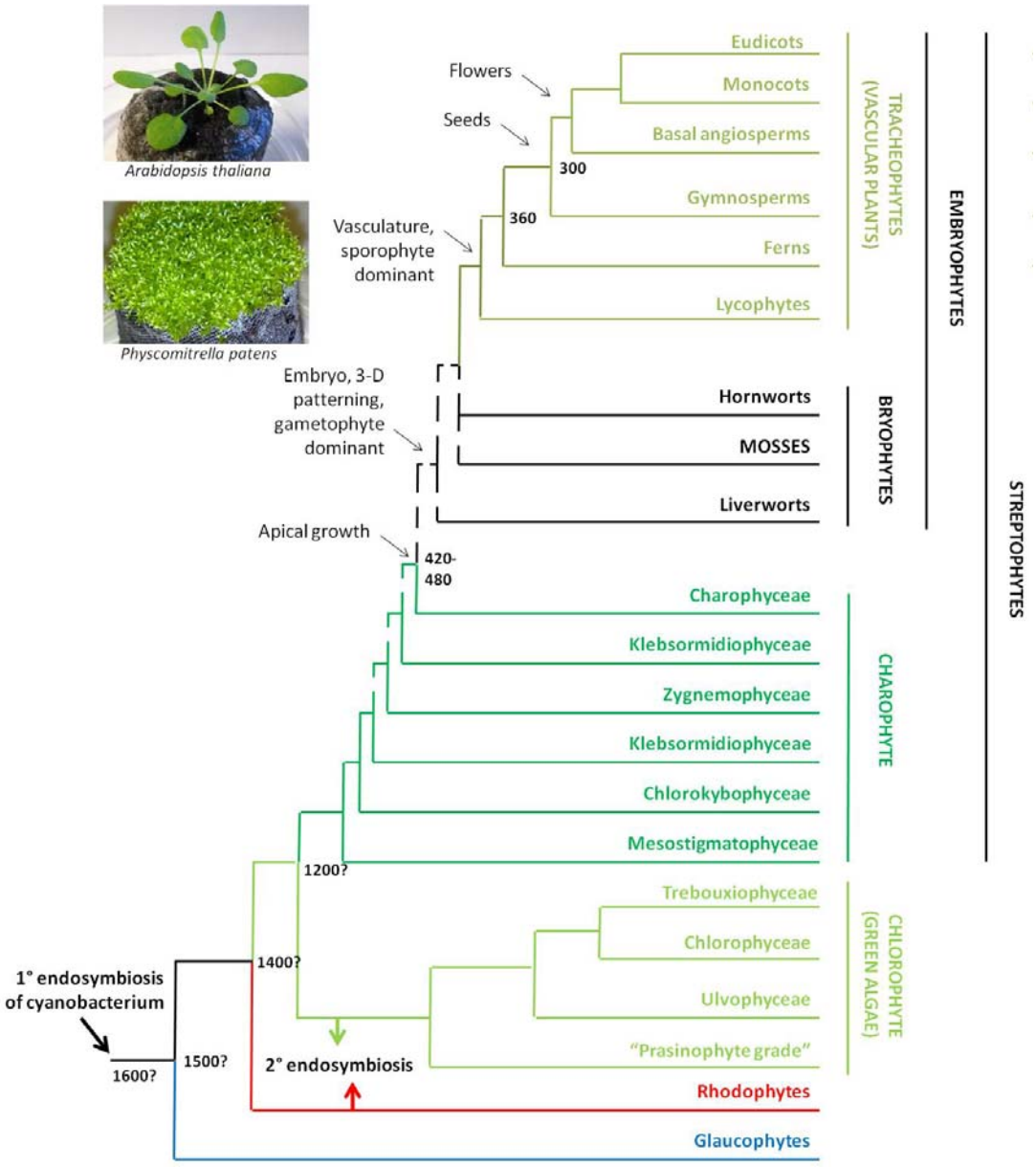

Figure 13. Phylogenetic relationships among Plantae. Divergence times for the nodes are different according to different estimations (Waters, 2003; Bowman *et al.*, 2007; Lang *et al.*, 2008). In addition, also the exact branching order of many taxa is still unknown (Waters, 2003), e. g. the branching order within the Charophyte algae (see also chapter 7 for a detailed discussion) or the one within the Bryophytes. Image adapted from (Bowman *et al.*, 2007). Photographs of the moss *Physcomitrella patens* and the model vascular plant *Arabidopsis thaliana* are also shown.

4.2. EVOLUTIONARY INTEREST IN MOSSES

As mentioned, Bryophytes (liverworts, mosses and hornworts) diverged from the tracheophytes (vascular plants), about 450 million years ago, in the early phases of land colonization by photosynthetic organisms. Because of this, they have an interesting evolutionary position and their comparisons with green algae and vascular plants may allow identify some of the adaptation of photosynthetic organisms during land colonization (Waters, 2003; Rensing *et al.*, 2008; Lang *et al.*, 2008).

Land plants had to adapt to the new and harsh condition of the terrestrial environment, such as the presence of UV radiations and strong illumination, faster oxygen diffusion and low availability of CO_2, flooding and dessication cycles, and different nutrient supply. Adaptation thus encompassed changes in morphology and in cellular, physiological and regulatory processes (Waters, 2003; Rensing *et al.*, 2008; Lang *et al.*, 2008).

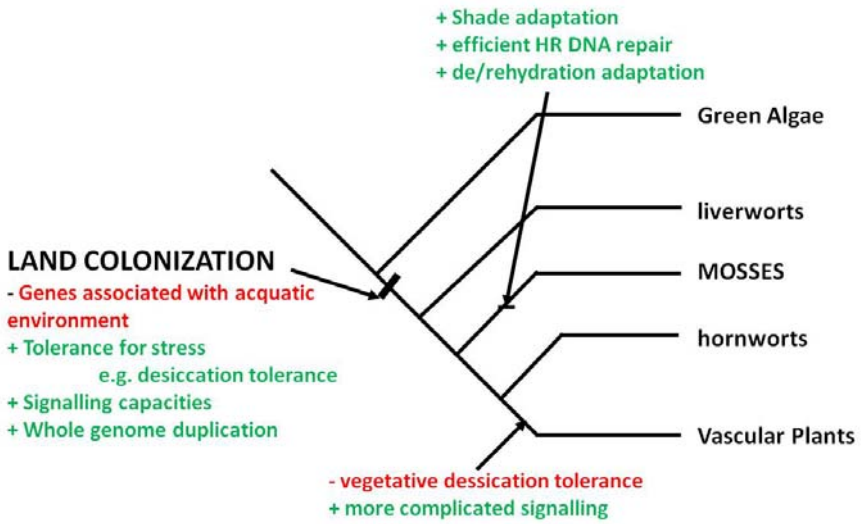

Figure 14. Land plant evolution. Key adaptation of the different groups of organisms are reported: differences of first land plants from algae, specific adaptations of mosses and also differences between mosses and vascular plants are shown. Some of these events may have been enabled by the opportunities for evolutionary novelty created by one of more duplications of the whole genome. Image adapted from (Rensing *et al.*, 2008)

The recent publication of a complete draft of a genome from a moss, *Physcomitrella patens*, (Rensing *et al.*, 2008) showed several changes occurring during the transition to land (figure 14).

In particular, compared to algae, the last common ancestor of all land plants lost genes associated with aquatic environment, while gaining key capacities for surviving on land. This leads, as examples, to signalling capacities (auxin, abscissic acid, cytokinin and more complex photoreception), tolerance for abiotic stress such as dessication and freezing tolerance, heat resistance, synthesis and accumulation of protective “sunscreens” (as

the presence of flavonoids), more elaborate transport capabilities, enhanced DNA repair mechanisms, an overall increase in gene family complexity and the development of an embryo within a multicellular reproductive organ (Waters, 2003; Glime, 2007; Rensing *et al.*, 2008).

Genomes comparison also allowed a reconstruction of the genomic events that occurred after the separation between vascular plants and mosses ancestors. In fact, they evolved and expanded with different strategies: while bryophytes developed a dominant gametophyte that still combined sexual reproduction with the availability of free water, vascular plants with their dominant sporophyte became more independent from water in their sexual reproduction. Compared to mosses, vascular plants acquired even more complicated signalling (e.g. gibberellic acid, jasmonic acid, ethylene, brassinosteroids), but lost vegetative dehydration tolerance and mobile gametes (Rensing *et al.*, 2008; Lang *et al.*, 2008).

4.3. THE MOSS *PHYSCOMITRELLA PATENS*

The moss *Physcomitrella patens* belongs to the family of *Funariaceae* in the order *Funariales* within the class *Broypsida* (Lang *et al.*, 2008): this species has received increasing attention in the recent years as a model organism. *P. patens*, like the model higher plant *Arabidopsis thaliana*, is a short lived (about 3 months life cycle) annual opportunist. It grows in late summer to autumn in temperate zones in an open distributed environment, often close to the waterline. Like other mosses, in fact, it is heavily dependent on water (flooding) for its reproduction but can survive to desiccation (Schaefer & Zryd, 2001; Cove, 2005; Lang *et al.*, 2008).

It is a monoecious, self-fertile plant. Its life cycle (figure 15) is dominated by the photoautrophic haploid generation, the gametophyte, which presents two different developmental stages (protonema and gametophore). Gametophytic generation supports a relatively simple and mainly heterotrophic diploid sporophyte.

Briefly, spores germinate to form protonemal tissue: it is a filamentous network of chloronemal and caulonemal cells, which develop by apical growth and cell division of apical and subapical cells. Chloronema cells are densely packed with large chloroplasts, while caulonema cells are characterized by an oblique cell wall and a small number of chloroplasts. Caulonema filaments gave rise to bud production, which involves a transition from two dimensional filament growth to three dimensional shoot development: the second gametophyte stage, called gametophore or leafy shoot, in fact, differentiates by caulinary growth from a simple apical meristem (the bud). The

gametophore is made up of a photosynthetic non-vascularized stem which carries the leaves and the reproductive organs, and of filamentous rhizoids that arise from the base of the stem. Fertilization gives rise to the diploid sporophyte, which comprises a seta, the stalk that bears the capsule in which spores are produced (Schaefer & Zryd, 2001; Cove, 2005).

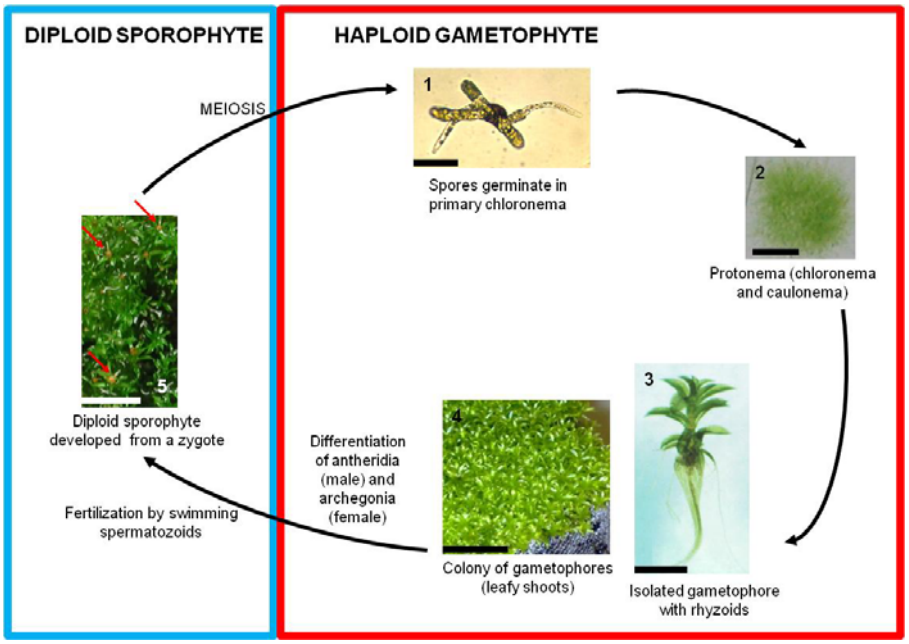

Figure 15. The life cycle of *P. patens*. Light-dependent spore germination generating primary chloronema (1). Protonemal colony (2), with branching chloronema and caulonema cells. Filamentous two-dimensional structure switches to three dimensions with the appearance of young buds in caulonema filaments. The bud develops to the leafy shoot of the gametophore (3). A fully developed moss gametophore (5 weeks old) (4). Differentiation of archegonia (female structures) and antheridia (male structures): both structures are present on the same plant as *P. patens* is monoecious. After fertilization by swimming spermatozoids (under water), the egg cell develops into a small diploid sporophyte (5) and within its capsule meiosis occurs leading to spore formation. The whole cycle can be achieved under optimal laboratory conditions in less than 12 weeks. Imagine adapted from (Schaefer & Zryd, 2001). Photographs 1 and 3 are from (Schaefer & Zryd, 2001). Bars: 1, 50 µm; 2, 1mm; 3, 0.5mm; 4, 1cm; 5, 0.5cm. red arrow: sporangia.

Physcomitrella can be easily cultivated in laboratory conditions both in liquid and solid media containing only inorganic salts. Cells from almost any tissue of *Physcomitrella*, both gametophytic and sporophytic, can regenerate to produce protonemal tissue, although culture are most quickly established starting from young protonemal tissue (Cove, 2000).

4.4. *PHYSCOMITRELLA PATENS* AS MODEL ORGANISM

The potential of mosses as model systems to study plant biological processes was already recognized some decades ago: as explained before, mosses are characterized by a dominant gametophytic generation, which facilitates genetic approaches, and present a relatively simple developmental pattern. They are suitable for cell lineage analysis and show similar response to plant growth factors and environmental stimuli as those observed in other land plant (Cove *et al.*, 1997). Plant physiologist focused their studies on some species, as *Funaria hygrometrica*, *Ceratodon purpureos* and *Physcomitrella patens*. The possibility to realize crosses *in vitro* led to the choice of *P. patens* for genetic approaches. Studies on *Physcomitrella* went on with the isolation of some auxotrophic mutants, and the isolation and characterization of biochemical and developmental mutants generated by chemical mutagenesis (reviewed in (Cove *et al.*, 1997; Schaefer & Zryd, 2001; Cove, 2005)).

Physcomitrella then emerged between other species in the Nineties as the first moss to be successfully transformed due to its capacity, unique among plants analyzed so far, to perform homologous recombination. *P. patens* shows levels of gene targeting comparable to those shown by the yeast *Saccharomyces cerevisiae* (Kammerer & Cove, 1996; Schaefer & Zryd, 1997; Hofmann *et al.*, 1999; Schaefer, 2001; Hohe *et al.*, 2004). Such an ability makes *Physcomitrella* a very powerful tool for plant functional and physiological studies since the generation of knock-out plants depleted in specific proteins, or the gene replacement with a mutated one to study proteins function, are highly facilitated. *Physcomitrella* has been widely used, for example, for metabolism and developmental studies (Cove, 2005; Cove *et al.*, 2006)

Due to the very interesting peculiarity of *Physcomitrella* to perform homologous recombination, in the recent years also many molecular genetics and genomics tools were developed for *P. patens* (Cove, 2005). Among them, the transformation methods have been further developed and optimized, allowing, for examples, the generation of multiple targeted gene KO (Hohe *et al.*, 2004) or the use of Cre/Lox system to excide undesired integrated sequences (Schaefer & Zryd, 2001). In addition, constitutive and inducible promoters (Schaefer & Zryd, 2001; Cove, 2005; Quatrano *et al.*, 2007) are available for this moss, allowing the over-expression of homologous and heterologous proteins (Quatrano *et al.*, 2007). Also RNA interference (RNAi) systems for a knock down of gene expression (Bezanilla *et al.*, 2003) have been developed. Moreover, many full-length cDNA, EST libraries and BAC-end sequencing are available (Rensing *et al.*, 2002;

Quatrano *et al.*, 2007), which recently led to the completion of the first draft of the complete genome sequence (see 4.2) (Rensing *et al.*, 2008) and later of the first physical linkage map of *Physcomitrella patens* (Kamisugi *et al.*, 2008).

Thus, its homologous recombination capacity, the availability of many experimental tools, the easy cultivation in laboratory conditions, together with its position in the phylogenetic tree, make *Physcomitrella* a unique model for plant functional genomics and for provide key insights in the evolution of land plants.

4.5. *PHYSCOMITRELLA PATENS* AS A NEW MODEL ORGANISM FOR PHOTOSYNTHESIS

4.5.1. PHOTOSYNTHESIS AND PHOTOPROTECTION IN MOSSES

As explained in sections 4.1 and 4.2, mosses are characterized by some morphological and physiological differences compared to vascular plants. Their photosynthetic apparatus is similar to green algae and vascular plants ones, with the presence of Chl *a* and *b*, xanthophylls and carotene as photosynthetic pigments and Lhc proteins for light harvesting (Glime, 2007; Alboresi *et al.*, 2008). Mosses often live in shade habitats, and thus they have to efficiently use the light which filter from the surrounding trees canopy. Consistently mosses are characterized by a "shade-adapted" photosynthetic apparatus, with Chl a/b ratio of about 1-2.5 depending on the species. Chl b is specifically bound to antenna complexes and these Chl a/b value are typically values found in shade adapted plants (Glime, 2007).

Another main difference with higher plants is the simpler leaf morphology: it is usually one-cell-thick and thus mosses are not able to respond to different light conditions by modifying leaves thickness, as seed plants do. In addition, despite the lack of stomata is a disadvantage in term of the inability to control their internal hydric status, this leads to the advantage of direct availability of water and CO_2 from the environment. Consistently, Bryophytes, which are C3 plants, are characterized by an higher CO_2 compensation points than tracheophytes, and are thus able to exploit the higher CO_2 concentration near the soil due to decomposition. This capacity allow mosses to take advantages from energy coming from "sunflecks", burst of bright light which filters from the canopy, to perform their photosynthesis (Glime, 2007).

Moreover, also mosses ability of protective energy dissipation has been studied, with particular attention to their response to dessication. Besides the presence of an efficient zeaxanthin-dependent energy dissipation, as in vascular plants, dessication-tolerant

bryophytes posses also specific dissipation mechanisms induced by structural alteration in chlorophyll proteins induced by dessication or based on stabilization of radicals within the reaction centre (these radicals are stable as long as water is absent). These mechanisms allow a rapid recovery of photosynthesis capacity upon rehydration (Heber *et al.*, 2006; Heber *et al.*, 2007).

4.5.2. *PHYSCOMITRELLA* AS A MODEL ORGANISM FOR PHOTOSYNTHESIS AND PHOTOPROTECTION

In the main part of this thesis we exploited the moss *Physcomitrella patens* as a model organism to study photosynthesis, focusing in particular on the analysis of the fast photoprotection process called Non Photochemical Quenching (NPQ).

This choice is justified by its position in the phylogenetic tree (see section 4.2) which allows clarifying the evolution of these mechanisms upon land colonization, which provided new challenges for photosynthetic organisms compared to water, and thus need the evolution of fast and efficient protection mechanisms to avoid photooxidative damage. The starting point for this thesis was the *in silico* and biochemical analysis of *Physcomitrella* antenna polypeptides by (Alboresi *et al.*, 2008). This study showed many interesting peculiarities of *Physcomitrella* photosynthetic apparatus. This work showed the presence of a “fuctional core” of antenna proteins conserved within the whole green lineage (Lhca1, lhca2, lhca3, lhcb4, lhcb5), while other proteins are specifically found in specific groups of organisms. Some of them (lhcb3 and lhcb6) are only present in land plants suggesting their role in adaptation of terrestrial environment. A new antenna polypeptide likely associated with PSII antennae, called Lhcb9, was also identified ((Alboresi *et al.*, 2008); see also appendix 1).

Concerning photoprotection mechanisms, sequence analysis highlighted the presence of both PSBS and LHCSR polypeptides (Alboresi *et al.*, 2008). As explained in section 3, PSBS protein is involved in vascular plant NPQ process (Li *et al.*, 2000), while LHCSR was recently recognized implied in NPQ in *C. reinhardtii* and diatoms (Peers *et al.*, 2009; Zhu & Green, 2010; Bailleul *et al.*, 2010). Thus, *Physcomitrella* was the first organisms were both the proteins responsible for NPQ in algae and vascular plants have been found expressed, making this moss a unique model to study NPQ process and its evolution from algae to land colonization.

This result was the starting point for the main part of this thesis: we exploited *Physcomitrella* ability of gene targeting to obtain mosses specifically depleted in PSBS, and/or LHCSR1 and LHCSR2 (the two LHCSR isoforms present in *Physcomitrella* genome), in order to comprehend their role in *P. patens* NPQ induction. In addition, we analyzed the physiological role of these different polypeptides during acclimation. We also generated over-expressor mosses for both PSBS and LHCSR in order to deeper analyze their activation mechanisms (see chapters 2-4). In addition to PSBS and LHCSR-dependent photoprotection, in order to obtain an exhaustive analysis of NPQ in this organism, we also evaluate the role of zeaxanthin in the thermal dissipation of *Physcomitrella* (chapter 6).

Moreover, we exploited *Physcomitrella* ability of doing homologous recombination to analyze *in vivo* the structure-function correlation in PSBS protein: we expressed deleted PSBS polypeptides in *psbs* KO background to clarify if the deleted regions are indeed necessary for PSBS activity, as reported in chapter 5.

At last, in order to obtain clearer information on the evolution of NPQ from algae to plants we also analyzed this process in some algae Streptophyte (chapter 7).

5. EXPERIMENTAL TECHNIQUES

Each chapter reports experimental methods employed. However, in this introductory paragraph are detailed the main experimental methods employed in this thesis: culture and transformation of *Physcomitrella patens* and the measurement of NPQ.

5.1. PROPAGATION OF *PHYSCOMITRELLA* IN LABORATORY CONDITIONS

Physcomitrella can be easily cultivated in laboratory conditions both in liquid and solid media containing only inorganic salts. In the latter case, solid media in Petri dishes is overlaid by a cellophane disk to provide mechanical support (A.A. PACKAGING LIMITED, PRESTON, UK). The cellophane disk is not necessary, but facilitate subsequent observation and collection of biological material. Cultures are grown in a growth chamber at 24°C, with 16 h light/8 h dark photoperiod and a light intensities of about 40-50 μE.

Growth and propagation of this moss is quite simple. Cells from almost any tissue of *Physcomitrella*, both gametophytic and sporophytic, can regenerate to produce protonemal tissue, although cultures grow faster if started from young protonemal tissue (Cove, 2000). In this work protonema tissue from 6-7 days-old plates are collected and tissue is blended in water by homogenizing moss material with Polytron (IKA T25 Digital Ultra Turrax). The suspension is then spread in new agar plates with medium overlaid with cellophane disks, where it regenerates rapidly as an uniform rug. This propagation protocol works well for short term cultures. In the growth conditions we used, cultures have to be analyzed and sub-cultured before a week of growth on rich medium, or about 2 in the case of minimum medium.

Homogenized tissue can be stored for some months at 4°C. Alternatively, long-term culture can be established: small explants of tissues can be singularly sub-cultured in a new agar plate with a needle to generate new clonal plants which are let growing slowly for few months. Moreover, *Physcomitrella* can complete all its life cycle also under lab conditions: collection of sporangia is the best method for the long term storage of mutant lines. To this aim, homogenized tissue is spread on sterile soil (jiffy, Jiffy Products International AS, Norway) instead of agar plates. After 5 weeks of growth in standard conditions (16/8 h photoperiod, 24°C) cultures are moved to 16°C and short day photoperiod (8h light/16 h dark) to induced antheridia and archegonia differentiation on gametophore shoot apices and fecundation: after about other 6-8 weeks mature

sporangia can be collected. Thus, in about 2-3 months the whole life cycle of *Physcomitrella* is completed (as exemplified in figure 16).
Protocols were adapted from the ones available at NIBB PHYSCObase (http://moss.nibb.ac.jp; see also refs therein) and (Asthon *et al.*, 1979).

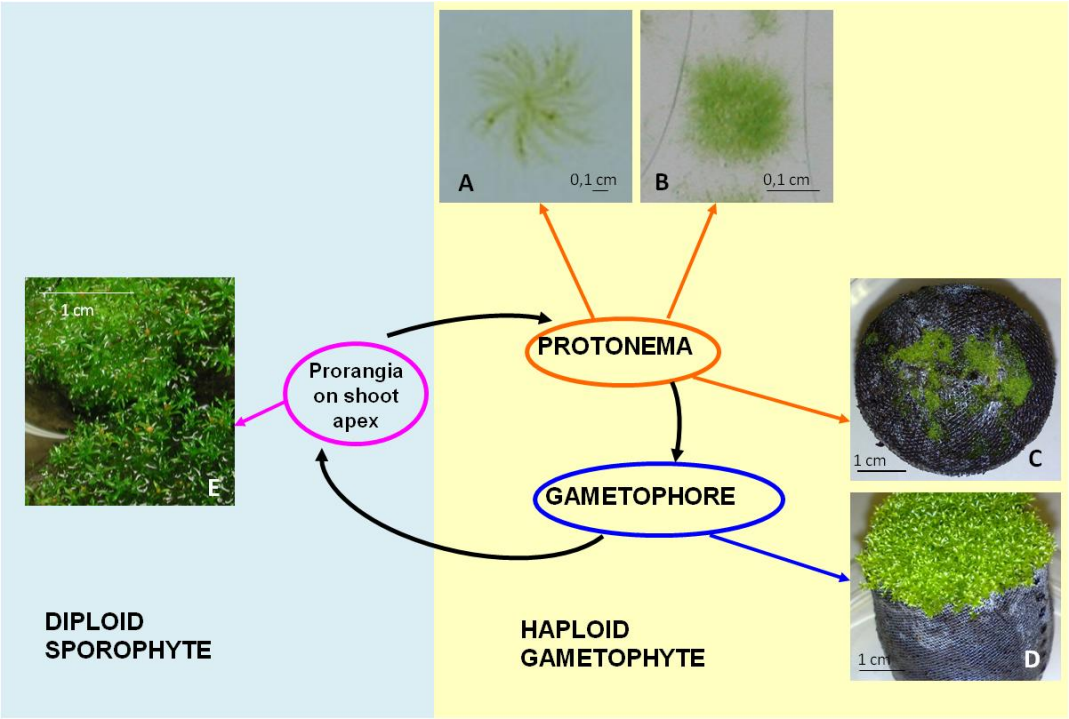

Figure 16. Examples of *P. patens* cultures: plants grown on minimal medium (A, 3 weeks old, propagated as tissue explants with needle), rich medium (B, 1week old, grown from tissue homogenized in sterile water) or sterile soil (C, 1 week old, protonema tissue; D, 5 weeks old, gametophore) at 40 µE m^{-2} s^{-1}. (E) gametophores with mature sporangia after 6 weeks at 16°C with short day photoperiod.

In this thesis, we characterized *Physcomitrella patens* (Gransden ecotype) in the protonemal stage of development grown on solid minimum medium ($PpNO_3$). Cultivation on agar plates with rich medium was used only to obtain starting culture for further analyses or for moss transformation, while mosses growth on sterile soil leads to the completion of the whole life cycle, going from protonemata up to fecundation and sporangia production.

5.2. TRASFORMATION OF THE MOSS *PHYSCOMITRELLA PATENS*

5.2.1. HOMOLOGOUS RECOMBINATION IN *PHYSCOMITRELLA PATENS*

As already reported in the section 4.4, *P. patens* present the unique ability among plants of doing homologous recombination, with a level of gene targeting comparable to those

shown by the yeast *Saccharomyces cerevisiae* (Kammerer & Cove, 1996; Schaefer & Zryd, 1997; Hofmann *et al.*, 1999; Schaefer, 2001; Hohe *et al.*, 2004). This ability allows specific gene knock-out by insertion or point mutation by gene conversion, also in the case of a specific member of a multigene family (Hofmann *et al.*, 1999), thus allowing for proteins functional analyses *in vivo*.

Integration of foreign DNA sequences in the genome by homologous recombination (HR) or illegitimate recombination (IR) seems to be tightly correlated with the dominant pathway used by cells to repair DNA double-strand breaks (DBS). Nevertheless, our knowledge of the mechanism by which HR occurs preferentially in *Physcomitrella* remains limited (Schaefer, 2001; Kamisugi *et al.*, 2005). However, many studies report key parameters to be optimized for a good yield of transformants with a targeted insertion of the transgene. Among them, the frequency of HR at the target locus is related to the length of the homologous sequences, showing that an homology region of about 1 kb is sufficient to achieve 50% of targeted transformants (Kamisugi *et al.*, 2005). Also the conformation of the plasmid is important, with linearized DNA showing the highest efficiency. The optimization of the DNA amount to be used for each transformation (10-20 μg) also improved the efficiency (Hohe *et al.*, 2004; Kamisugi *et al.*, 2005; Kamisugi *et al.*, 2006). Simultaneous transformation with multiple vectors to obtain a multiple targeted KO has also been reported, although the probability of obtaining a multiple KO with a targeted gene replacement in all desired loci with a single transformation is low, being the combination of the frequencies observed for individual constructs (Hohe *et al.*, 2004; Kamisugi *et al.*, 2006).

In addition to precise targeted gene replacement (TGR) with the integration of a single copy of foreign DNA construct at the target site, allele replacement may also involved insertion of multiple copies of the transgene, accompanied by ectopic insertions at non-homologous site, as detailed reported in figure 17, and also insertions at multiple loci may occur. It is worth mentioning, however, that in each case presented in figure 17, HR takes place in at least at one end of the targeting construct. This reinforce the view that HR is the major pathway by which transforming DNA is integrated in *Physcomitrella*, instead of the most common non-homologous end joining (NHEJ) found in many organisms (Schaefer, 2001; Kamisugi *et al.*, 2005; Kamisugi *et al.*, 2006).

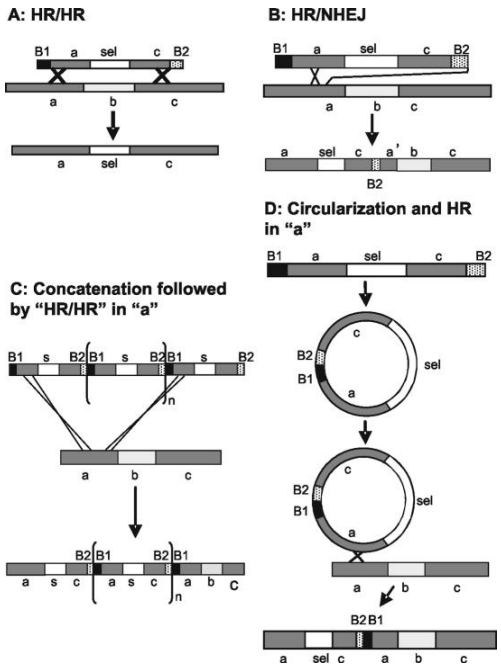

Figure 17. The targeting vector for the gene "a-b-c" present sequences homologous to "a" and "c" genomic regions, while the central segment "b" is replaced by a selectable marker cassette "sel". B1 and B2 are distinct, non-homologous, cloning vector-derived sequences **A.** The transforming fragment integrates by HR in both arms of the vector, and a perfect TGR occurs. **B.** The transforming fragment integrates by HR in one arm of the vector: the other arm invades the breakpoint to integrate by NHEJ (TI, Targeted Insertion). **C.** The transforming fragment undergoes head-to-tail concatenation prior to integration. Integration occurs in region "a" of the targeted locus by two HR events occurring in separated regions "a" in the concatemer. "s" and "sel" indicate the *npt*II selection cassette. **D.** The transforming fragment is circularized by NHEJ. Recombination at a single point in region "a" generates a transgenic locus indistinguishable from that in "C". Image from (Kamisugi *et al.*, 2006)

5.2.2. PEG-MEDIATED TRANSFORMATION OF PHYSCOMITRELLA PATENS PROTOPLASTS

Transformations reported in this work were performed as in (Schaefer & Zryd, 1997) with minor modification. Protoplasts for moss transformation are obtained from 5-6 days old protonema inoculated from a freshly fragmented culture. Protoplasts are isolated from protonema by the digestion (30 min at RT) with 1% (w/v) Driselase (Sigma-Aldrich) dissolved in 8.5% (w/v) mannitol. The digested moss material was then filtered by gravity through a 100µm sieve, left for additional 15min at room temperature to continue digestion on the filter and carefully washed with 8.5% mannitol. The protoplasts were collected by centrifuging the filtrate at 200g for 5 minutes at room temperature, then supernatant was discarded and the pellet was washed twice in 8.5% mannitol.

Protoplasts were then resuspended in MMM solution (8.5% mannitol, 15mM $MgCl_2$, 0.1% MES, pH 5.6) at a concentration of $1.2*10^6$ cells/ml. Afterwards, aliquots of 15µg of linearized DNA was dispensed in falcon tubes and 300µl of protoplast suspension and

300µl of PEG solution (7% mannitol, $Ca(NO_3)_2$ 0.1 M, PEG 4000 35-40%, 10 mM Tris pH 8) were added mixing gently. The mixture was heat-shocked 5 minutes at 45°C and brought to room temperature for 10 minutes. Samples were progressively diluted with liquid rich medium ($PpNH_4$ supplemented by 66g/l mannitol; adding 5x300 µl and 5x1000 µl) and incubate in dark at room temperature overnight.

The next day, protoplasts were embedded in protoplast top-layer (mannitol 8.5% with 0.84% agar Sigma (A9799); 7 ml of top layer for each tube, thus final agar concentration is 0.42%) and plated on the cellophane covered plate with rich medium ($PpNH_4$) added with 66g/l mannitol and 0.7% agar.

Plates were incubated in a plant growth chamber under standard growth conditions (16/8 h photoperiod, 24°C, light intensity about 40 µE). Selection of transformants started 6 days after transformation by transferring top layer to a new Petri dish with $PpNH_4$ medium supplemented with the appropriate antibiotic (50 µg/ml G418, 30 µg/ml hygromycin or 50 µg/ml zeocin) for about 10 days. Resistant colonies are then transferred to non-selective $PpNH_4$ medium for additional 10 days, and then again on selective media to isolate only stable transformants which integrated the transgene in their genome. In fact, *Physcomitrella* is also able to episomally replicate exogenous circularized DNA. This can be the original vector if transformation was performed with the circular plasmid, or mainly a circularized concatenamer of transgene fragments in the case of transformation with linearized DNA (Ashton *et al.*, 2000; Muren *et al.*, 2009). These lines represent “transient transformants”, as the extra-chromosomal elements are then likely lost during growth in non selective media (Ashton *et al.*, 2000; Muren *et al.*, 2009), and thus these lines are not able to survive to the second selection or show mosaicism for the resistance.

5.2.3. PLASMIDS EMPLOYED FOR *PHYSCOMITRELLA* TRANSFORMATION

In this thesis we presented results both from knock-out (KO) mosses as well as from over-expressor (OE) lines. We employed different strategies in the two cases: for the generation of targeted KO plants we cloned region of the genome flanking the gene of interest to obtain the substitution of the coding sequence with resistance cassette. In the case of OE we targeted the coding sequence of the protein to a region of *Physcomitrella* genome (called BS213) known to have no gene expression and not to the natural gene locus. In this case, thus, the expression of the protein is under the control of a constitutive promoter (called 7113) and thus it is not physiologically regulated by its own promoter.

In the case of KO generation we employed different vectors carrying different antibiotic resistances, to have the possibility of combine different mutations in multiple KO lines. Regions from PSBS (locus XM_001778511), LHCSR1 (locus XM_001776900), and LHCSR2 (locus XM_001768019) genes were cloned into BZRf/BNRr, BHRf, and BNRf plasmids (kindly provided by F. Nogue, Institut National de la Recherche Agronomique, Versailles, France), respectively. These plasmids present a very similar backbone with different resistance cassettes (the example of BZRf is reported in Figure 18A). In all cases 5' and 3' sequences of the target gene were cloned in the Multi Cloning Sites (MCS) at the two end of the resistance cassette, to obtain the substitution of the coding sequence with the antibiotic resistance, as exemplified in figure 18B for BZRf vector. Detailed procedure used to clone flanking regions of PSBS, LHCSR1 and LHCSR2 genes is reported in chapter 2, table S1.

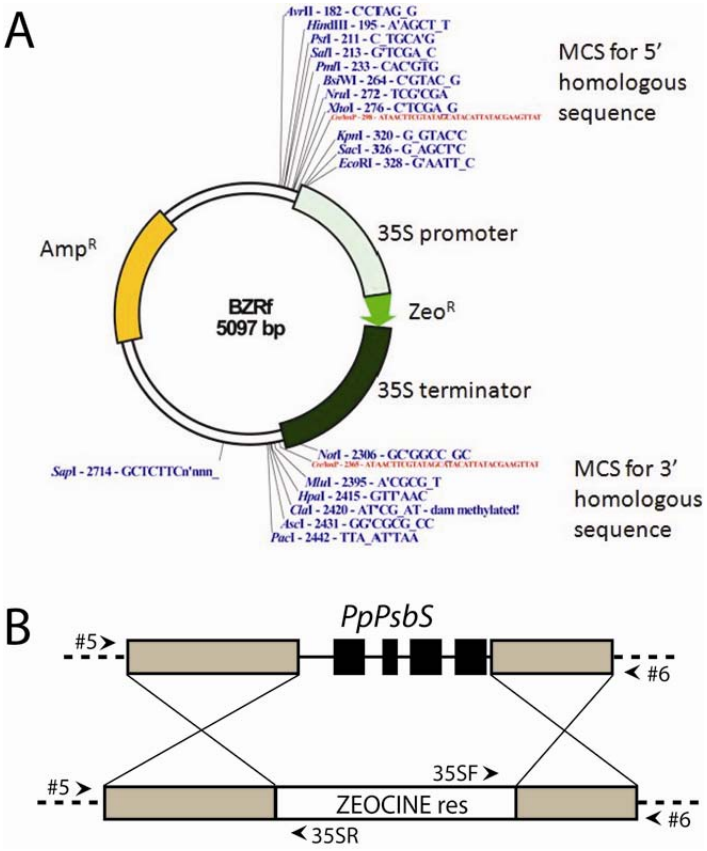

Figure 18. A) Example of vector used for KO generation: BZRf plasmid, used for the generation of *psbs* KO mutants. The other vectors used for KO generation in chapter 2 and 4 (BNRr, BHRf, and BNRf) present a very similar design. In orange is reported Ampicillin resistance, used for the propagation of the plasmid in *E. coli*. In green arrow zeocin resistance, with 35S promoter upstream and 35S terminator downstream. Some restriction sites are reported in blue, with attention to multicloning sites (MCS) used for the integration of 5' and 3' genomic homologous regions. Cre/lox sites are also reported in red. B) Integration of the transgene in *Pp* genome. The genomic region of PSBS gene is schematized, with exons shown in black. Gray boxes represent the genomic regions exploited for homologous recombination. Below is shown the constructs for homologous recombination: genes for antibiotic resistance are located between regions homologous to the genome. #5, #6, 35SF and 35SR are primers employed to verify DNA insertion (see chapter 2).

For the OE generation, instead, we employed the pMAK1 vector, available at NIBB PHYSCObase (http://moss.nibb.ac.jp) and kindly provided by Prof. Takashi Murata (National Institute for Basic Biology, Okazaki, Japan). In this case, both the coding sequence of the protein of interest and the selection cassette (zeocin resistance) are comprised between the two sequences homologous of BS213 region of the genome (see figure 19). Thus, after HR both the coding sequence for our protein and the antibiotic resistance are integrated in the genome.

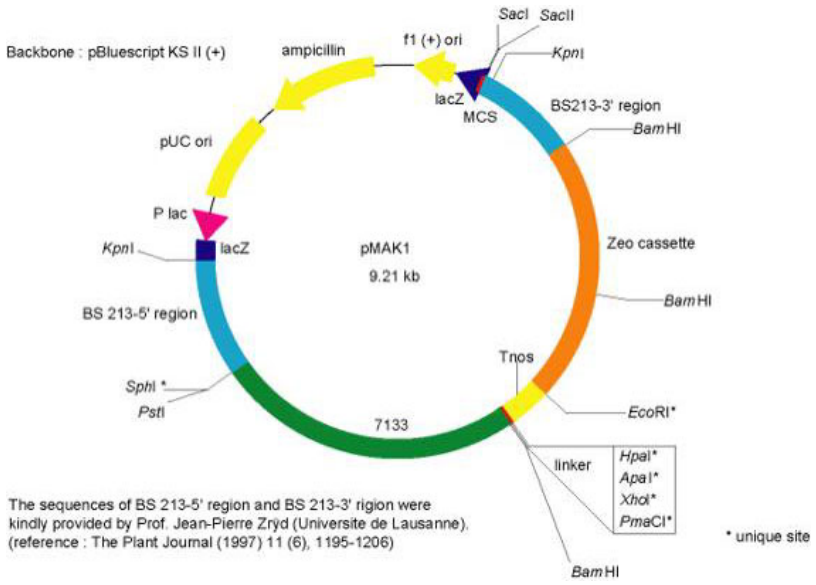

Figure 19. pMAK1 vector. In bright yellow bacterial plasmid derived sequences. In bright blue sequences homologous of BS213 region, in green the constutive promoter (7133 in the figure; later it was found that this promoter was 7113) which control the expression of the gene of interest, then the multicloning site (linker, in red) to insert the coding sequence and the terminator Tnos, in yellow. In orange is reported zeocin cassette. Image from NIBB PHYSCObase (http://moss.nibb.ac.jp/).

We cloned the coding sequences (cDNA) of *Physcomitrella* PSBS (XM_001778511.1) (entire coding sequence of with N- or C-terminal deletions, see chapter 4 and 5) or LHCSR1 (XM_001776900.1) (chapter 4) using the multicloning site ("linker" in figure 19) of pMAK1. Primers and restriction enzymes employed to obtain each transforming vector are reported in table 1. DNA obtained from *E. coli* was digested with *KpnI* restriction enzyme prior to *Physcomitrella* transformation.

Primer name	Sequence
PSBS_FOR	CGCTCGAGATGGCTCAAGCTGCACTCATTTC
PSBS_REV	CGGTTAACTCAGTCCTCGATGTCGTCGTCG
PSBS_ΔCtermREV	CGGTTAACTCAGTCGTTGACGAACTTGCCAGTTCC
PSBS_pepREV	GGATGTCCCAAAAATAAACAAGGCGAAGGTCTTCACAGC
PSBS_ΔNterFOR	GACCTTCGCCTTGTTTATTTTTGGGACATCCGGTGGG
LHCSR1_FOR	CGCTCGAGATGGCGATCGCTATGTCCTCCG
LHCSR1_REV	CGGGGCCCTTACAGGCCCAATCTCTTGAACAAATGC

Table I. Primers employed for amplification and cloning of *Physcomitrella* PSBS or LHCSR1 coding sequences from cDNA. Restriction enzyme sites are reported as underlined. Primers PSBS_FOR and PSBS_REV were employed to obtain the entire coding sequence of PpPSBS protein. Instead, amplifications with PSBS_FOR and PSBS_ΔCtermREV lead to PSBS protein deleted in its C-terminal aminoacids (see chapter5). To obtain the N-terminal deletion, we first separately obtain the sequences of the transit peptide which direct the protein to thylakoids (primers PSBS_FOR and PSBS_pepREV) and of the mature protein without the N-term aa (primer PSBS_ΔNterFOR and PSBS_REV). The primers PSBS_pepREV and PSBS_ΔNterFOR and designed to anneal one to the other: this allows to obtain a "fusion" of the two amplicons in a further PCR using the two fragments as templates and PSBS_FOR and PSBS_REV as primers: the obtain amplificate thus present the transit peptide fused with PSBS coding sequence shortened in the N-terminus (see chapter 5). Each of these PSBS coding sequences was then cloned into pMAK1 by digestion with XhoI/HpaI endonucleases. To obtain LHCSR1 coding sequence PCR was performed with LHCSR1_FOR and LHCSR1_REV primers and cloned into pMAK1 using XhoI/ApaI restriction sites.

5.2. *IN VIVO* CHLOROPHYLL FLUORESCENCE TO MEASURE NPQ USING THE PAM FLUOROMETER.

As discussed in section 2 of the introduction, solar light is harvested by pigments bound to Lhc antenna proteins. Light absorption results in singlet-excited state of Chl *a* molecules (^{1}Chl*), which can return to the ground state by different pathways (Figure 20). Excitation energy, in fact, can be re-emitted as Chl fluorescence, it can be transferred to reaction centers and used to drive photochemistry (qP), or it can be de-excited by thermal dissipation processes (NPQ). If these three de-excitation mechanisms are not able of dealing with all excitation energy, singlet states can convert into triplet states (^{3}Chl*) by intersystem crossing. Since all different de-excitation pathways start from the same excited state the relative yield of fluorescence can change depending on the efficiency of the other pathways, qP and NPQ. (Niyogi, 1999; Niyogi, 2000; Muller *et al.*, 2001).

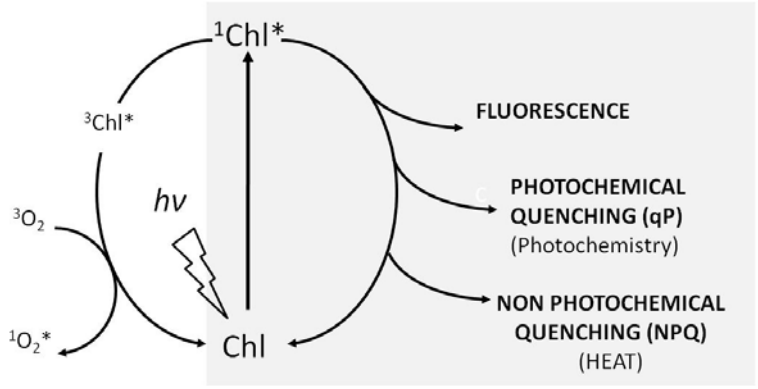

Figure 20. Pathways of excited ^{1}Chl* molecules de-excitation. Image modified from (Muller *et al.*, 2001).

As a consequence, monitoring Chl fluorescence can give information about NPQ and photochemical quenching. The use of Chl *a* fluorescence measurements to examine photosynthetic performances in algae and plants is widely used since several years for physiological studies. At room temperature, Chl fluorescence originated majorly from PSII. This is monitored using fluorometers called PAM (Pulse Amplitude Modulation) which are capable of monitoring fluorescence in dark adapted and illuminated leaves using different frequency modulated light sources. Three different lights are employed for this analysis:

- Measuring light (FML): weak and pulsed light, too low to induce any photosynthetic electron transfer. This is the light source employed to monitor fluorescence. Thanks to its modulation (switched on and off at high frequency) the detector is capable of detecting only the fluorescence originating from this measuring light also in the presence of other lights.
- Actinic light (AL): this light is absorbed by the photosynthetic apparatus and drives electron transport. Its intensity has to be optimized for each species. Usually, to analyze NPQ actinic light intensity is adjust in order to almost saturate photochemical capacity of the sample, to achieve also the maximal NPQ induction. In fact, plants are able to maintain a low steady-state fluorescence yield and ^{3}Chl* yield due to a combination of qP and NPQ.
- Saturation pulse (SP): brief (<1 s) pulse of light that completely saturates photochemistry so that there is no quenching anymore due to qP, and thus the quenching due solely to NPQ can be determined.

In figure 21 is reported the fluorescence quenching chart of a typical induction kinetic protocol. Samples are dark adapted before measurement to ensure full oxidation of all electron transporters. After this dark period, thus all PSII reaction centers are “open”, thus ready to use a photon for photochemical reduction of Q_A. In the presence of the

measuring light, the minimal (F_0) fluorescence of the sample is determined. On the contrary, the first SP allows determining the maximal fluorescence (Fm). SP is short pulse with very high intensity which reduces all Q_A sites; as a consequence all PSII reaction centers are "closed" and fluorescence reaches its maximal value. SP has very short time length and thus its contribution to photochemistry is negligible. After a short period of dark adaptation (usually 30-60 seconds, according to the species) to allow re-oxidation of electron transporters, actinic light is turned on to analyze Chl fluorescence during illumination. Upon onset of actinic illumination, fluorescence yield first quickly rises to a peak, which is followed by a biphasic decline (Kautsky effect). The period of actinic light is usually follow by a period of dark, where the relaxation kinetics of the processes previously induced by light exposure can be analyzed. During all the experiment, repetitive SPs allow to determine the different contribution of photochemical quenching and NPQ, thanks to the determination of local maximum of fluorescence (Fm'), as graphically reported in figure 21 (Maxwell & Johnson, 2000; Baker, 2008). As reported above, in fact, fluorescence quenching is due to both photochemistry and thermal dissipation. To distinguish between these two contributions the usual approach is to "switch off" one of the two contributions, specifically photochemical quenching, so that the fluorescence yield in the presence only of the other (NPQ) can be estimated: SP transiently close all reaction centers, and during the flash the fluorescence yield reaches a value equivalent to that which would be attained in the absence of any photochemical quenching (Maxwell & Johnson, 2000; Baker, 2008).

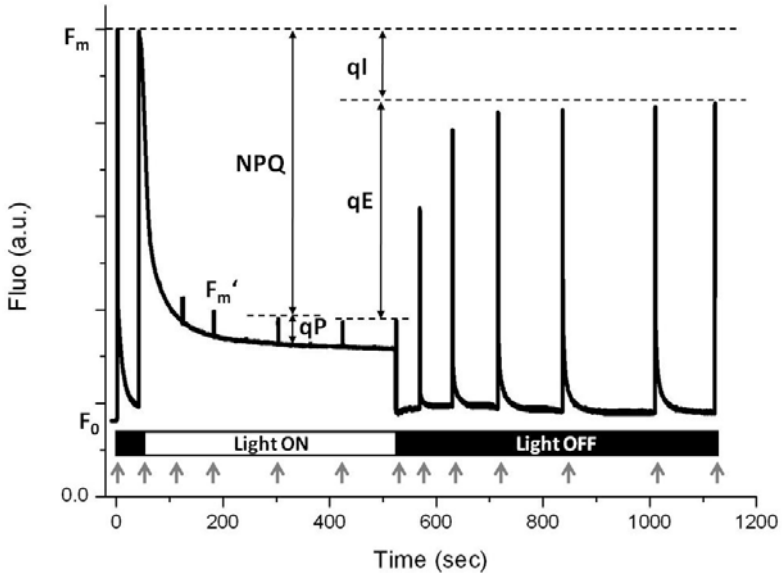

Figure 21. Typical fluorescence chart of *Arabidopsis thaliana*. Measuring light is on throughout all the analysis time. Black and white bars: actinic light OFF/ON. Grey arrows: saturation pulses (SP). F_0, Fm, Fm' are indicated. Graphical view of photochemical quenching (qP) and non photochemical quenching (NPQ and its components qE and qI) are also reported. Adapted from (Muller *et al.*, 2001).

Many different parameters can be derived by the examination of fluorescence chart obtained by PAM fluorometer. Here I present only the ones widely used in this thesis (Fv/Fm and NPQ), but a large number of different coefficients have been calculated to quantify photochemical and non photochemical quenching (Demmig-Adams *et al.*, 1996; Maxwell & Johnson, 2000; Kramer *et al.*, 2004; Baker, 2008).

One of the main parameter derived from fluorescence analysis is F_v/F_m ratio:

$$F_v/F_m = (F_m-F_0)/F_m$$

It is the ratio between variable fluorescence and maximal fluorescence of dark adapted sample, which is used to estimate the maximum quantum yield of PSII. This value is usually about 0.8 for plants, and a decrease in this parameter is usually related to stress conditions and radiation damage to PSII. Thus, F_v/F_m is also a simple and rapid way of monitoring stress.

The overall NPQ of Chl fluorescence can be determined as

$$NPQ = (F_m – F'_m)/F'_m$$

This formula does not discriminate between qE and qI contributions. Thus, in this thesis we referred as qE for the rapid relaxing component of NPQ as seen from its kinetic, while qI was the part of thermal dissipation which not was reverted during the analysis dark period (10 min), as usually reported in the definitions of qE and qI in literature (Niyogi, 1999; Niyogi, 2000; Szabo *et al.*, 2005) and as shown in figure 21 and figure 9 of this introduction.

In addition to the induction curve presented above, also light curve protocols can be used to investigate the response of a plant to increasing (and/or decreasing) light intensities. Even in this case the different parameters can be determined by SP at the end of each light step.

For our analyses on *Physcomitrella* samples we employed a Dual-PAM-100 fluorometer (Walz, Germany). We optimized the conditions to measured reproductively NPQ in this organism. The standard induction curve protocol used in our analyses was: 40 seconds of dark recovery after F_0/Fm determination, which ensures a complete relaxation of fluorescence yield induced by the SP, 8 min of actinic light period with SP every 20 seconds and 10 min of dark recovery. Measuring light was set at 46 µE, actinic light at 830 µE, while saturation pulse was of 6000 µE and 600 ms long. The analyses were done after a dark adaptation of the samples of 40 minutes.

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CHAPTER 2

PHYSCOMITRELLA PATENS MUTANTS AFFECTED ON HEAT DISSIPATION CLARIFY THE EVOLUTION OF PHOTOPROTECTION MECHANISMS UPON LAND COLONIZATION

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PHYSCOMITRELLA PATENS MUTANTS AFFECTED ON HEAT DISSIPATION CLARIFY THE EVOLUTION OF PHOTOPROTECTION MECHANISMS UPON LAND COLONIZATION

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ABSTRACT

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 as to avoid oxidative damage. Non Photochemical 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 knock out 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.

INTRODUCTION

Sun light 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 physico-chemical 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 single states ^{1}Chl* (6-8). NPQ has two major components: energy quenching (qE), activated within seconds upon an increase in light intensity, and inhibitory quenching (qI), which is slower and relaxes within 1-2 hours 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 states 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 the field conditions wherein plants lacking PSBS are rapidly counter-selected (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 so far 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), while other algae at most show stacking between two thylakoid membranes.

Studying organisms which diverged from the green lineage leading to vascular plants early after land colonization helps us to understanding how photosynthetic organisms adapted to the challenges of different environmental conditions. The moss *Physcomitrella patens* is a valuable choice as 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.

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 a Lhc-like proteins has been found in several algae but not in land plants, with the remarkable exception of *P. patens*, a bryophyte which 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 accumulates in the membrane of thylakoids (Fig. 1*A*). A search in *Arabidopsis* and *Chlamydomonas* 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.

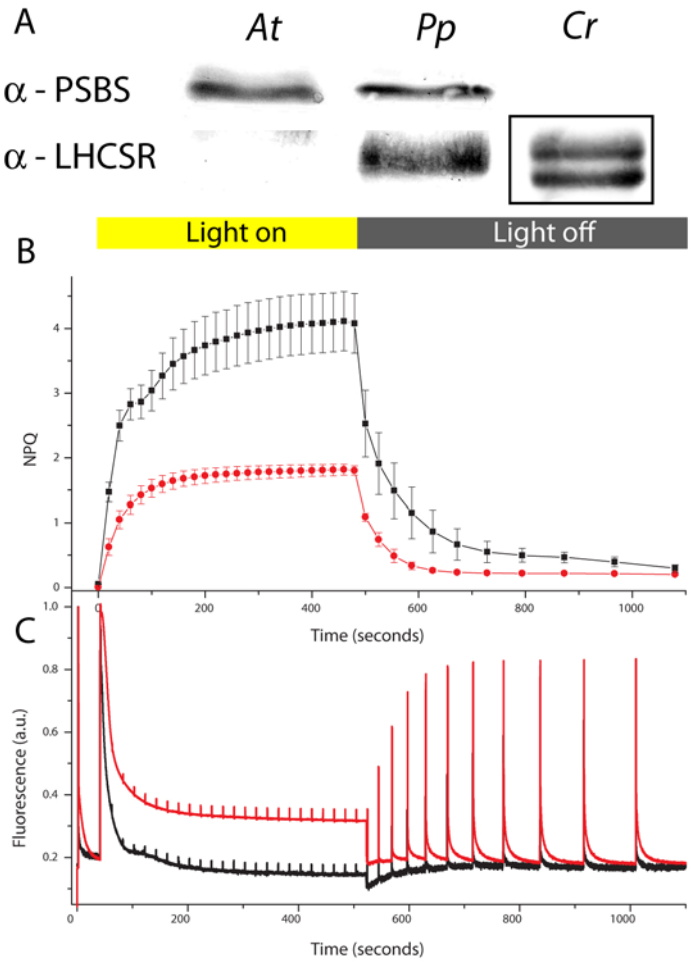

Fig. 1. Comparison of NPQ *A. thaliana*, *P. Patens,* and *C. reinhardtii*. (A) Western blotting against NPQ involved polypeptides, PSBS and LHCSR in *Arabidopsis* (At), *Physcomitrella* (Pp) and *Chlamydomonas* (Cr). Thylakoids (1 µg of Chl) was loaded in all cases. In the case of Cr, thylakoids were 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 weight 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 (Fm'), and thus activate maximal NPQ.

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 (Fm'), 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 1C). 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. 1200 µE m^{-2} s^{-1}), capacity of heat dissipation was higher in *P. patens* than in *Arabidopsis*. 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 knockout 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 knockout mosses depleted in the three genes encoding putative NPQ triggering polypeptides. *P. patens* is a very valuable organism for gene knockout 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 protoplast transformation leaded 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 (example of amplificates 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 assays with primers annealing to the genomic sequences at either sides 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. 2*E*). 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. 2*F*).

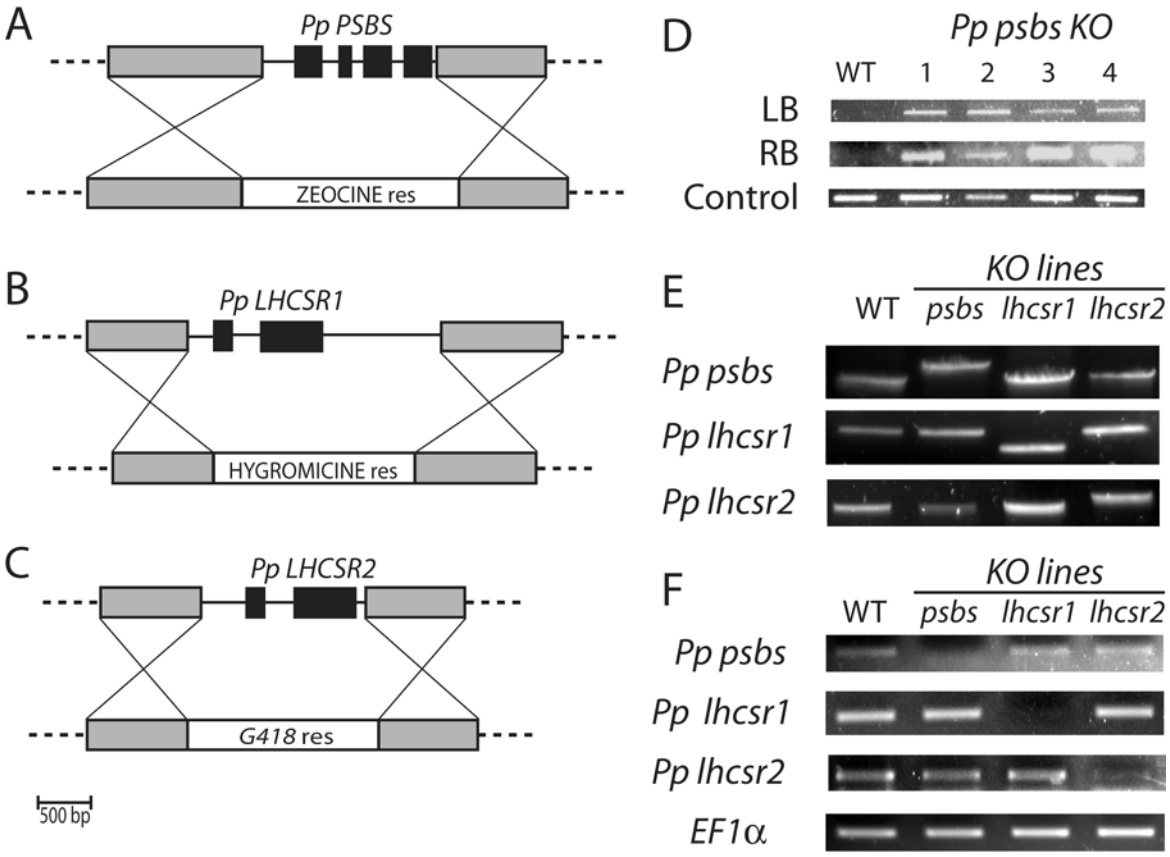

Fig. 2. KO mutant generation and characterization. Scheme of constructs used for KO generation. Genomic region of *PSBS* (*A*) and *LHCSR* (*B* and *C*) genes is schematized, with exons shown in black. Gray boxes represent the genomic regions exploited for homologous recombination. Below are shown the 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 (*EF1α*) is also reported as control.

Single insertion mutants were grown and 10 days old plants were harvested and used for thylakoid purification. When analyzed by western blotting, no signal for PSBS was detected in *psbs* KO (Fig. 3*A*). Knockout for *LHCSR* showed that most of immunoblotting signal was lost in *lhcsr1* KO whereas protein level was substantially unaffected in *lhcsr2* KO. Red Ponceau staining and anti-CP43 antibody was routinely used to verify equal loading for all samples (Fig. 3*A*). 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 photosynthetic apparatus which is thus comparable in all mutants. The maintenance of photosystems organization was also confirmed by measuring the functional antenna size, as determined *in vivo* by measuring fluorescence 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 Fv/Fm value of 0.80 ± 0.03 after 10 days of growth in control light conditions.

	WT	***Psbs KO***	***lhcsr1 KO***	***lhcsr KO***
Chl a/Chl b	2.56	2.58	2.52	2.60
Chl/ Car	4.33	4.25	4.60	4.43
	WT	***psbs lhcsr ko***	***psbs lhcsr.1 lhcsr2 ko***	
Chl a/Chl b	2.56	2.42	2.57	
Chl/ Car	4.33	4.52	4.29	

Table 1. Pigment binding properties of isolated mutants. Pigment binding properties of single and multiple mutants depleted in PSBS, LHCSR1 and LHCSR2, respectively. Chl a/ b and Chl / car ratio are reported. Standard deviation is below 0.1 in the case of Chl a/ b and 0.3 for Chl/Car.

When NPQ kinetics were recorded, instead, knockout plants showed large differences with respect to WT (Fig. 3*B*). The stronger phenotype was observed in the case of *lhcsr1* KO, followed by *psbs* KO and *lhcsr2* KO. This pattern is consistent with western blotting analysis shown in Fig. 3*A*: most LHCSR protein immuno-detected in WT is synthesized by *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.

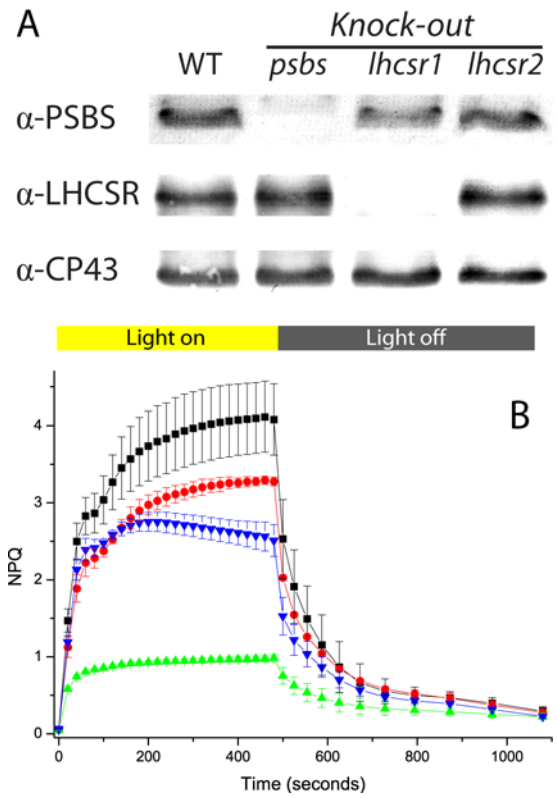

Fig. 3. *P. patens psbs* and *lhcsr* KO phenotype. **(***A*) Western blottings using antibodies against LHCSR and PSBS. A western blotting against CP43 is also shown as loading control. Thylakoids were loaded in all cases, 0.5 µg of Chl for anti-PSBS and 0.3 µg for all the others. (*B*) NPQ kinetics 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 5 independent measures.

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 lumenal pH, from its fast kinetics of relaxation and sensitivity to nigericine. 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 NPQ phenotype, implying that the low NPQ phenotype was attributable to the KO mutations (Fig. S5). Moreover, transformations using two different plasmids, carrying either zeocine or neomicyn 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 blotting as described above (Fig. 4*A*). 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 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. 4*B*). In fact, the small residual NPQ was stable during at least 10 minutes of dark recovery following illumination, as observed in the PSBS-less *npq4* mutant of *Arabidopsis* (10).

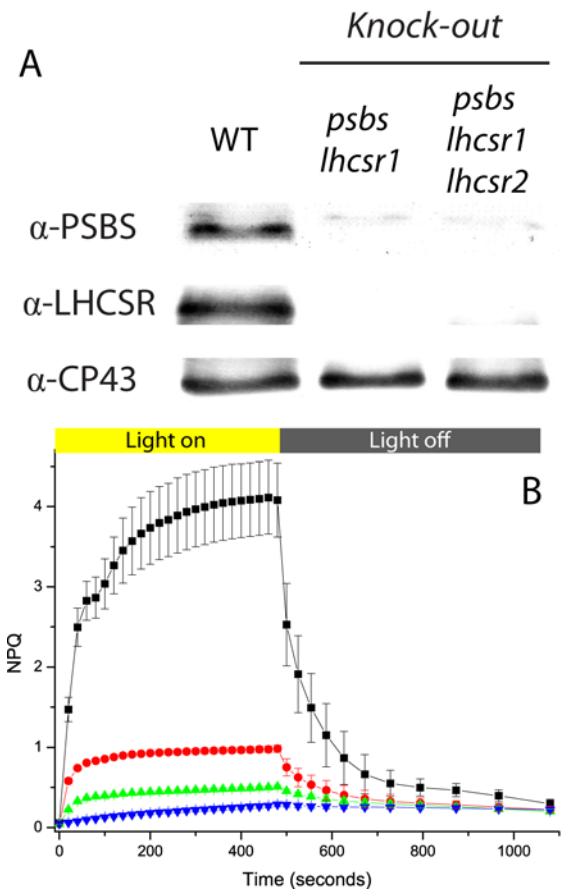

Fig. 4. Phenotype of *P. patens psbs* and *lhcsr* KO double and triple mutants. **(***A*) western blotting using antibody against LHCSR and PSBS. (*B*) NPQ kinetics of selected lines. Curves for WT, *lhcsr1* KO, *psbs lhcsr*1 KO, and *psbs lhcsr*1 *lhcsr2* KO are shown in black, red, green and blue, respectively. Averages and SD are calculated from at least 5 independent measures.

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 mutants 5-day-old plants with high light intensities. Fv/Fm was measured every day to monitor PSII photodamage. As shown in figure 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 lhcrsr2* 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 these plants show partial recovery over the following days, demonstrating that other acclimating mechanisms acting with slower time courses are active in mosses independently from 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.

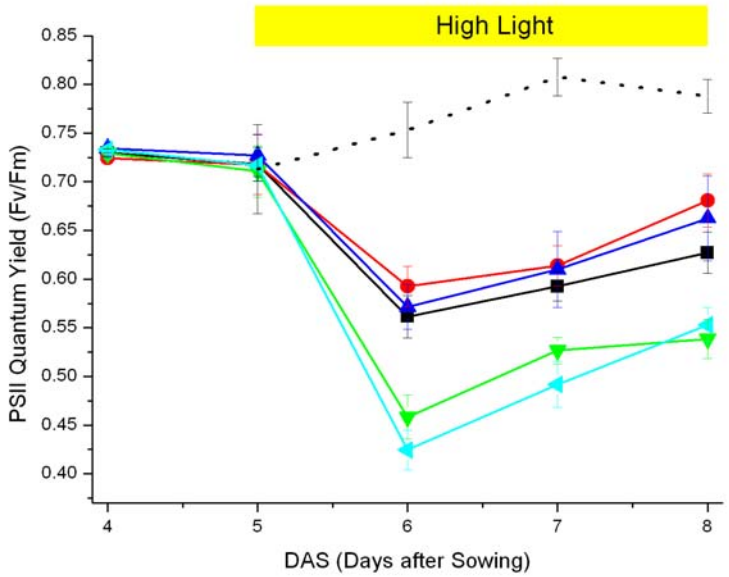

Fig. 5. Photoprotection capacity of NPQ affected mutants. The photoprotection capacity of the previously described mutants hasbeen compared with WT by treating 5-day-old plants with additional 3 days of high light. PSII quantum yield expressed as Fv/Fm was monitored daily. WT, *psbs* KO, *lhcsr1* KO are shown in black squares, red circles and blue triangles, respectively , whereas *psbslhcsr1* and *psbslhcsr1lhcsr2* KO double and triple mutants are shown in green triangles and cyan triangles. 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 undistinguishable from data on WT.

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 *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 an 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 role 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 they are present in other mosses as well. There is no indication at present that any vascular plant carries 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 here that mutants lacking PSBS or most of 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, consistentl 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 mutants 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, in addition, the newly evolved PSBS-dependent mechanism typical of land plants. Together, these mechanismsprovide optimal photoprotection of *Physcomitrella* in high light conditions. Our results suggest that 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 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 counter-selection 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).

MATERIAL AND METHODS

Plant Growth. Protonemal tissue of *P. patens*, Gransden wild-type strain was grown on minimum $PpNO_3$ 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^{-2} s^{-1} (control conditions). For high light tests, 5-day-old plants were moved from control to 350 µE m^{-2} s^{-1}, maintaining temperature and photoperiod.

NPQ measurements. In vivo chlorophyll fluorescence in *P. patens* was measured of room temperature with Dual PAM-100 fluorometer (Heinz Walz GmbH, Germany), with a saturating light at 6000 µE m^{-2} s^{-1} and actinic light of 830 µE m^{-2} s^{-1}. In the case of *Arabidopsis*, plants were grown at 100 µE m^{-2} s^{-1}, in an 8-h light/16-h dark photoperiod

and were measured with 1200 µE m^{-2} s^{-1} of actinic light. Before measurements, plates were dark-adapted for 40 minutes 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, INRA Versailles), 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 ml^{-1} G418 (Sigma-Aldrich); 30 µg ml^{-1} Hygromicyn B (Sigma-Aldrich) or 50 µg ml^{-1} zeocin (Duchefa Biochemie)). Resistant colonies were transferred for 10 days on non-selective media and then again in antibiotic media to isolate stable transformants. Confirmation of DNA insertion was performed by PCR, as detailed in Table S1.

Thylakoids 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 nitro-cellulose membrane (Sartorious AG) using a blot system from Biorad and were detected with specific polyclonal antibodies produced in the laboratory.

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AUTHORS CONTRIBUTION

AA, RB, TM designed research; AA, CG performed experiments; AA, CG, TM Analyzed data; TM, GMG, RB wrote the paper.

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SUPPLEMENTARY MATERIAL

Table S1. **Primers employed for KO generation and screening.**

Gene	Primer Name	Sequence	USE
PpPSBS	PpPSBS#1	TGTGGTCGACCTCCAAATTAAGGTTTCGGGG	KO isolation
	PpPSBS#2	TGAGAGGCGTACGGCAGAGG	KO isolation
	PpPSBS#3	AACTCTAACCGCGGACGACACATTCTG	KO isolation
	PpPSBS#4	TCACAACTAGTAACACCAAG	KO isolation
	PpPSBS#5	TTGACATGGGAATGAACGTG	KO screening
	PpPSBS#6	TAGCGAGAACTGTACAGATG	KO screening
	PpPSBS#7	CTTTGGGTGACCGTGGAA	RT-PCR
	PpPSBS#8	ACACGGGGCCCTTTTCAT	RT-PCR
PpLHCSR1	PpLHCSR1#1	TTTAAGCTTGCTAGGCCTCC	KO isolation
	PpLHCSR1#2	ATTGCGTACGCCTCATCGTG	KO isolation
	PpLHCSR1#3	TAACCAGATCTCTTAACTCCC	KO isolation
	PpLHCSR1#4	ATCGGCGCGCCATCAACTAGTACAAAGCAAGAAATC	KO isolation
	PpLHCSR1#5	CAAGCTTTGACAACAACCACA	KO screening
	PpLHCSR1#6	TTGGATCTTAGAAGTGCAAGAAA	KO screening
	PpLHCSR1#7	CCCCAGCAGCCTACCAGGTG	RT-PCR
	PpLHCSR1#8	AGGCAGCACGAGCCAACAAA	RT-PCR
PpLHCSR2	PpLHCSR2#1	GGCACGTGGTTAGCTTGTTGACC	KO isolation
	PpLHCSR2#2	GCTCCCCGTACGTCTCTCTCGC	KO isolation
	PpLHCSR2#3	TTGAACCGCGGATTTTTTGTGATCC	KO isolation
	PpLHCSR2#4	TTCGCGGCCGCAGGCGCGCCAACCC	KO isolation
	PpLHCSR2#5	CAAGCTTTGACAACAACCACA	KO screening
	PpLHCSR2#6	TTGGATCTTAGAAGTGCAAGAAA	KO screening
	PpLHCSR2#7	TTGCAGCTGCACCTCACGCA	RT-PCR
	PpLHCSR2#8	CGTGAGTGATTTCCGACTCC	RT-PCR
35S-CaMW	35SF	CGCTGAAATCACCAGTCTCTCT	KO Screening
	35SR	GTGTCGTGCTCCACCATGT	KO screening
PpEF1α	PpEF1α#1 *	GCCAAGAAGAAGTGAATAGTGCG	RT-PCR
	PpEF1α#2 *	ACGTCTGCCTCGCTCTAGC	RT-PCR

* Primer Sequences were previously described by Lunde et al (1) (PMID: 16429263)

In the case of *PpPSBS* (XM_001778511), 1485 bp of the upstream region was amplified with PpPSBS#1 (5'-TGTGGTCGACCTCCAAATTAAGGTTTCGGGG-3') and PpPSBS#2 (5'-TGAGAGGCGTACGGCAGAGG-3') and cloned in BNRr by SalI and BsiWI to generate PpPSBSKO5' plasmid. Afterward, 1044 bp of the downstream region was amplified with PpPSBS#3 (5'-AACTCTAACCGCGGACGACACATTCTG-3') and PpPSBS#4 (5'-TCACAACTAGTAACACCAAG-3') and cloned in *PpPSBS* KO5' by SacII and SpeI to generate *PpPSBS* KO plasmid. Protoplasts were transformed with *PpPSBS* KO cut by SalI and SpeI. Zeocine resistance was subsequently introduced in this *PpPSBS* KO from BZRf by XhoI SacII endonucleases. For screening, PpPSBS#5 (5'-TTGACATGGGAATGAACGTG-3') and PpPSBS#6 (5'-TAGCGAGAACTGTACAGATG-3') were used with primers 35SF (5'-CGCTGAAATCACCAGTCTCTCT-3') and 35SR (5'-GTGTCGTGCTCCACCATGT-3'). In the case of *PpLHCSR1* (XM_001776900), 965 bp of the upstream region was amplified with PpLHCSR1#1 (5'-TTTAAGCTTGCTAGGCCTCC-3') and PpLHCSR1#2 (5'-ATTGCGTACGCCTCATCGTG-3') and cloned in BHRf by HindIII and BsiWI to generate PpLHCSR1KO5' plasmid. Afterward, 1170 bp of the downstream region was amplified with PpLHCSR1#3 (5'-TAACCAGATCTCTTAACTCCC-3') and PpLHCSR1#4 (5'-ATCGGCGCGCCATCAACTAGTACAAAGCAAGAAATC-3') and cloned in *PpLHCSR1* KO5' with NotI to generate

PpLHCSR1 KO plasmid. Protoplasts were transformed with *PpLHCSR1* KO cut by HindIII and MluI. For screening *PpLHCSR1* #5 (5'-CAAGCTTTGACAACAACCACA-3') and *PpLHCSR1* #6 (5'-TTGGATCTTAGAAGTGCAAGAAA-3') were used with 35SF and 35SR. In the case of *PpLHCSR2* (XM_001768019), 971 bp of the upstream region were amplified by *PpLHCSR2*#1 (5'-GGCACGTGGTTAGCTTGTTGACC-3') and *PpLHCSR2*#2 (5'-GCTCCCCGTACGTCTCTCTCGC-3') and cloned in BNRr by PmlI and BsiWI to generate *PpLHCSR2*KO5' plasmid. Afterwards 961 bp of the downstream region were amplified by *PpLHCSR2*#3 (5'-TTGAACCGCGGATTTTTTGTGATCC-3') and *PpLHCSR2*#4 (5'-TTCGCGGCCGCAGGCGCGCCAACCC-3') and cloned in *PpLHCSR2*KO5' by SacII and AscI to generate *PpLHCSR2*KO plasmid. Protoplasts were transformed with *PpLHCSR2*KO cut by PmlI and AscI. For screening *PpLHCSR2*#5 (5'-CAAGCTTTGACAACAACCACA-3') and *PpLHCSR2*#6 (5'-TTGGATCTTAGAAGTGCAAGAAA-3') were used with 35SF and 35SR.
For RT-PCR, the annealing temperature used for *PpLHCSR1* and *PpLHCSR2* primers was 65°C while in the case of *PSBS* it was 55°C.

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Fig- S1. Comparison of NPQ activation in *P. patens* and *A. thaliana* using different actinic light intensities. NPQ dependence from actinic light was compared in *P. patens* (black squares) and *A. thaliana* (red circles). Measurement was performed by treating with 60 s of each actinic light intensity before measuring NPQ using the light curve protocol from Dual PAM-100.

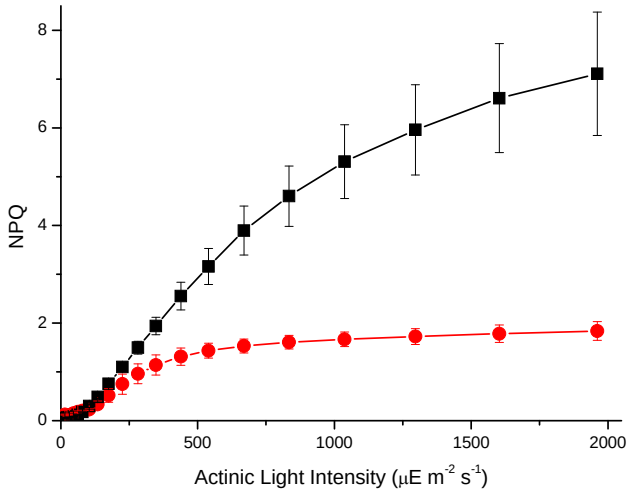


Fig. S2. Evaluation of antenna size in mutants analyzed in the present work. Functional antenna size was evaluated in all mutants by quantifying the fluorescence in DCMU-treated plants. Antenna size was determined as $1/t_{2/3}$.

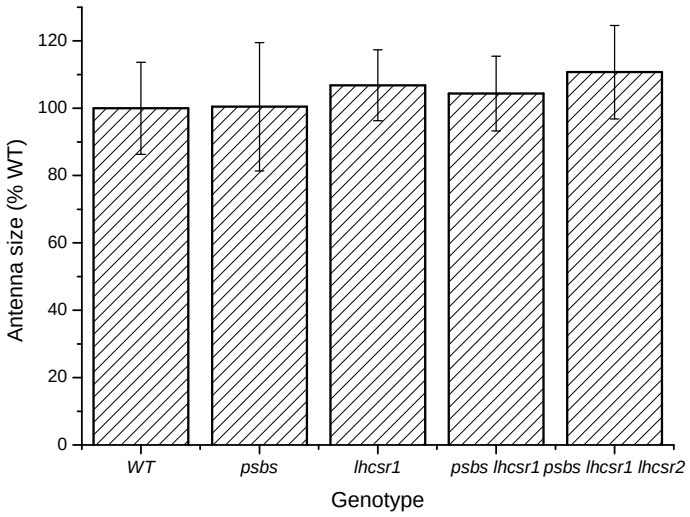


Fig. S3. Detection of LHCSR2. Western blotting against LHCSR in WT, *lhcsr1* KO and *lhcsr2* KO plants. Thylakoids (3 µg of Chl) have been loaded for each sample, thus ten times more than in figure 3.

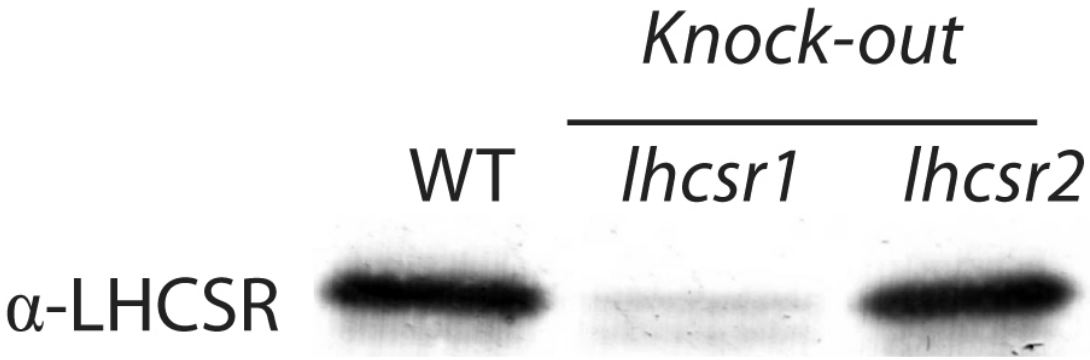


Fig. S4. NPQ kinetics of *Pplhcsr1* KO vs. *Arabidopsis*. NPQ kinetics of *A. thaliana* and *P. patens* WT (green and black, respectively) from Fig. 1 are compared with *Pplhcsr1* KO (red) from Fig. 3. Actinic light intensities used for *P. Patens* and *Arabidopsis* were as in Fig. 1.

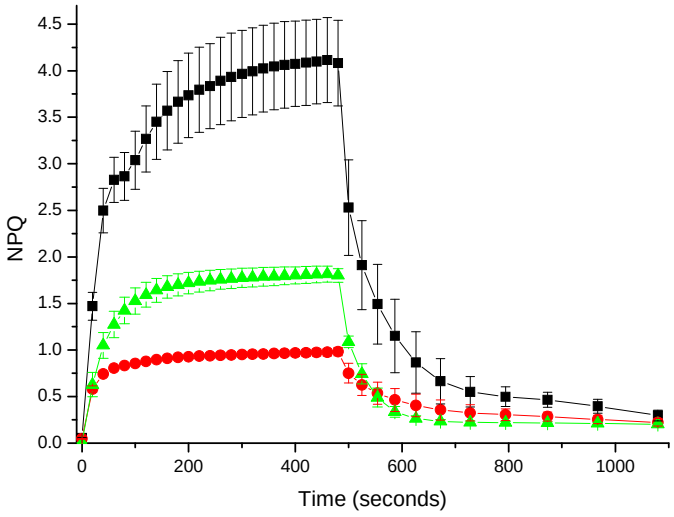


Fig. S5. NPQ kinetics from several independent lines from *lhcsr1* KO transformation are indicated with their clone number. NPQ values are slightly different from the one s reported in Figs. 1-3 because of the different growth medium and colony age.

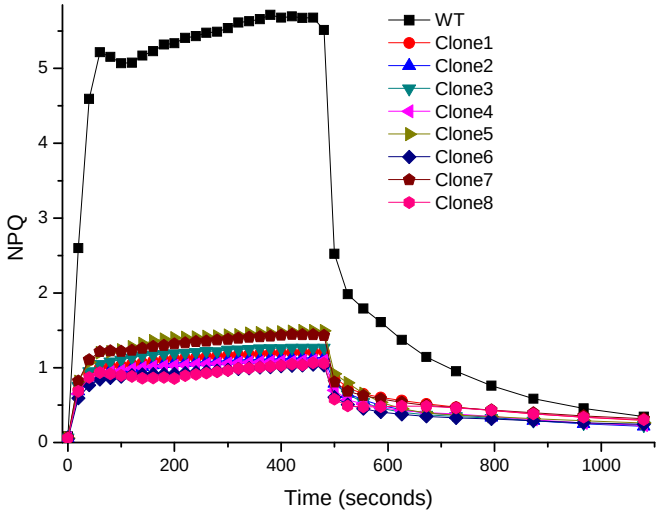
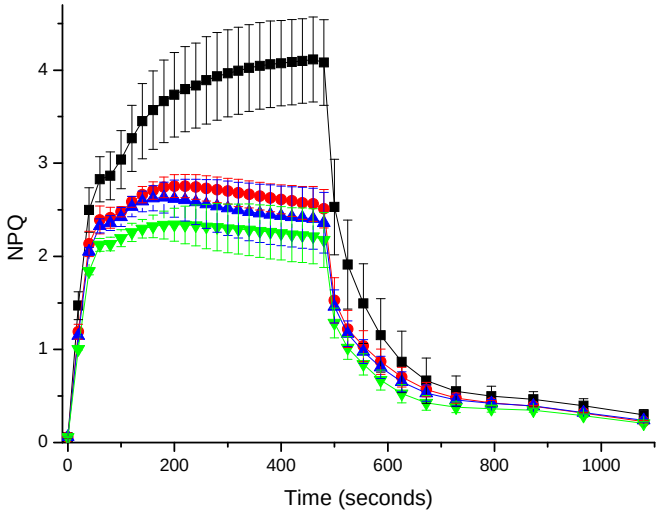


Fig. S6. NPQ kinetics of different lines of *psbs* KO resulting from different transformation events and different constructs. WT is shown in black, whereas three different lines of *psbs* KO are shown in red, blue and green. Blue and red were transformed with the construct schematized in Fig. 2*A*, whereas the green with a similar construct carried G418 resistance.



CHAPTER 3

ROLE OF PSBS AND LHCSR IN *PHYSCOMITRELLA PATENS* ACCLIMATION TO HIGH LIGHT AND LOW TEMPERATURE

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ROLE OF PSBS AND LHCSR IN *PHYSCOMITRELLA PATENS* ACCLIMATION TO HIGH LIGHT AND LOW TEMPERATURE

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ABSTRACT

Photosynthetic organisms respond to strong illumination by activating several photoprotection mechanisms. One of them, Non Photochemical Quenching (NPQ), consists in the thermal dissipation of energy absorbed in excess. In vascular plants NPQ relies on the activity of PSBS, while in the green algae *Chlamydomonas reinhardtii* it requires a different protein, LHCSR. The moss *Physcomitrella patens* is the only known organism in which both proteins are present and active in triggering NPQ, making this organism particularly interesting for the characterization of this protection mechanism. We analyzed the acclimation of *Physcomitrella* to high light and low temperature, finding that these conditions induce an increase in NPQ correlated to over-expression of both PSBS and LHCSR. Mutants depleted of PSBS and/or LHCSR showed that modulation of their accumulation indeed determines NPQ amplitude. All mutants with impaired NPQ also showed enhanced photosensitivity when exposed to high light or low temperature, indicating that in this moss the fast-responding NPQ mechanism is also involved in long-term acclimation.

Keywords: Photoprotection, photosystem, photosynthesis, Non Photochemical Quenching, NPQ, acclimation, mosses, plants evolution, *Physcomitrella patens*

INTRODUCTION

Oxygenic photosynthesis enables organisms to convert solar light into chemical energy but the complex redox activity of photosystem II (PSII) in the presence of oxygen may easily drive the production of reactive oxygen species (ROS) leading to oxidative damage of the photosynthetic apparatus (photoinhibition)(Murata et al. 2007;Szabo, Bergantino, & Giacometti 2005). This phenomenon becomes particularly relevant when the radiation absorbed exceeds the saturation level of photosynthesis (Barber & Andersson 1992;Demmig-Adams & Adams 2000;Li et al. 2009). Photosynthetic organisms evolved the ability of responding to variations in light intensity and they maximize the light harvesting efficiency to support photosynthesis when solar radiation is limiting. On the contrary, when light exceeds photosynthetic capacity, efficient dissipation of energy excess is activated thus minimizing ROS production and photoinhibition. Low temperature is known to play a synergistic role with excess illumination by limiting electron transport and carbon fixation rates. In these conditions, even a relatively weak light may exceed energy utilization rate with a consequent photoinhibitory effect (Huner, Oquist, & Sarhan 1998).

Changes in light intensity may occur with different timescales spanning from seconds, when clouds or the overlaying canopy interfere with the incident sunlight, up to weeks, as with seasonal changes. For this reason plants evolved multiple protection mechanisms, with different activation timescales, enabling response to the short- and long-term variations in environmental cues (Eberhard, Finazzi, & Wollman 2008;Huner et al. 1998;Walters 2005). The fastest among them is Non Photochemical Quenching (NPQ), which consists in the thermal dissipation of excess absorbed energy and is activated within seconds upon a change in light intensity. Interestingly, NPQ is present in both plants and green algae and yet its activation relies on different gene products: while the PSBS protein is crucial in plants, the green alga *Chlamydomonas reinhardtii* requires a distinct Lhc-like polypeptide called LHCSR (Li et al. 2000;Peers et al. 2009). In the timescale of several minutes, strong illumination also activates the synthesis of the xanthophyll zeaxanthin from pre-existing violaxanthin by activation of violaxanthin de-epoxidase (VDE) (Arnoux et al. 2009;Jahns, Latowski, & Strzalka 2009). Zeaxanthin, besides busting NPQ and retarding its relaxation in the dark, also plays a direct role in ROS scavenging (Demmig-Adams et al. 1990;Havaux, Dall'Osto, & Bassi 2007;Niyogi, Grossman, & Björkman 1998). On a longer-term, plants respond to changing light intensity by activating an acclimation response consisting in the modulation of gene expression, protein content and physiological properties to cope with the changing environmental conditions (Eberhard et al. 2008;Walters 2005). As an example, the concentration of carbon fixing enzymes increases in high light acclimated plants,

allowing for a better exploitation of the available radiation. Other responses commonly observed in plants are the reduction of PSII antenna size and pigment per area content (Bailey et al. 2001;Bailey, Horton, & Walters 2004;Ballottari et al. 2007;Melis 1991).

Physcomitrella patens is a moss which has received an increasing attention as a model organism for the study of the evolution of photosynthetic organisms. Mosses diverged from vascular plants early after land colonization and their study can highlight the events leading to adaptation to the harsher physico-chemical conditions of the sub-aerial ecosystem (Rensing et al. 2008). In land environment the concentration of oxygen, an inhibitor of photosynthesis, is higher (Scott & Glasspool 2006), while carbon dioxide, the final acceptor of electrons extracted from water, is generally less available. Moreover, the sessile form of life acquired on land prevents escaping strong changes in light intensity by swimming deeper, a behavior typical of algae (Waters 2003). The combination of these factors makes more likely the possibility that light is harvested in excess with respect to the maximal rate of photochemical reactions, thus making fast and efficient photoprotection responses essential for survival. An additional reason of interest is that in *P. patens* NPQ depends on the contribution of both PSBS and LHCSR proteins for its activation (Alboresi et al. 2010) and that this moss shows larger NPQ efficiency compared to the model organism for higher plants *Arabidopsis thaliana* (Alboresi et al. 2010).

In this study we analyzed the effects of acclimating *P. patens* to high light (HL) or low temperature (LT) on NPQ. We report that NPQ activity in HL grown *Physcomitrella* is enhanced through an increased accumulation of both PSBS and LHCSR. By analyzing KO mosses depleted in PSBS and/or LHCSR, we show that the level of each of these gene products indeed determine the amplitude of NPQ in different light and/or temperature conditions. The KO mutants also show an increased photoinhibition under strong illumination implying that NPQ, despite being a fast-activated process, also plays a role in long-term photoprotection in *P. patens.*

MATERIALS AND METHODS

Plant material and treatments. Protonemal tissue of *P. patens*, Gransden wild-type strain and *psbs* and/or *lhcsr* KO lines (Alboresi et al. 2010) were grown on minimum $PpNO_3$ (Asthon, Grimsley, & Cove 1979) 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, PRESTON, UK) as previously described (Alboresi et al. 2008). Plates were placed in a growth chamber under controlled conditions: 24°C, 16 h light/8 h dark photoperiod and a light intensity of 45 µE m^{-2} s^{-1} (control light conditions, CL). For light treatments, 5 days old plants were transferred to

10 µE m^{-2} s^{-1} (low light, LL) or to 450 µE m^{-2} s^{-1} (high light, HL). For cold temperature treatments, 5 days old plants were transferred to 4°C with 16 h light/8 h dark photoperiod and a light intensity of 45 µE m^{-2} s^{-1} (Low Temperature, LT)

NPQ measurements. In vivo chlorophyll fluorescence in *P. patens* (10 days old plants) was measured at room temperature with Dual PAM-100 fluorometer (Walz), using a saturating light at 6000 µE m^{-2} s^{-1} and actinic light of 830 µE m^{-2} s^{-1}. Before measurements, plates were dark-adapted for 40 minutes at room temperature. The parameters Fv/Fm, qL and NPQ were calculated as (Fm-Fo)/Fm, [(Fm'-F)/(Fm'-Fo') * Fo'/F] and (Fm-Fm')/Fm'. For inhibitors treatments *P. patens* colonies were incubated with 30 µM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) or 50 µM Nigericin in 150 mM sorbitol, 10 mM HEPES (pH 7.5) buffer during the 40 minutes dark-adaptation at RT before measurements. Control samples were incubated with the HEPES/sorbitol buffer only. Data are presented as mean ± SD of 3-6 independent experiments. Transmembrane potential was estimated from carotenoid electro-chromic shift (ECS) using a JTS-10 from BioLogic (France). Light adapted WT and the triple *psbs lhcsr1 lhcsr2* KO plants ECS signal was measured for 30 seconds of illumination at 940 µE m^{-2} s^{-1} and then for additional 30 seconds. Transmembrane potential was estimated from the decrease in ECS when light was switched off (Bailleul et al. 2010;Takizawa et al. 2007). To normalize the signal to the sample amount effectively illuminated, ECS were normalized to the maximal fluorescence measured in the same setup after cells were left 20 minutes in the dark to allow for quenching relaxation.

Thylakoids extraction, SDS-PAGE and Western Blotting analyses. Thylakoids from protonemal tissue (10 days old plants) were prepared using an *Arabidopsis* protocol with minor modifications (Alboresi et al. 2008). For immunoblotting analysis, following SDS-PAGE, proteins were transferred onto a nitro-cellulose membrane (Pall Corporation) using a blot system from Amersham and detected with specific home-made polyclonal antibodies.

RESULTS

Acclimation of Physcomitrella patens to different light intensities.

Five days old *P. patens* plants were grown for five additional days at three different light intensities: low light (LL, 10 µE$m^{-2}s^{-1}$), control light (CL, 45 µE$m^{-2}s^{-1}$) and high light (HL, 450 µE$m^{-2}s^{-1}$). Up to 10 days cultures in all conditions are composed only of protonema, while after 11-12 days plants start developing visible three dimensional gametophores. This was also influenced by light conditions (Thelander, Olsson, & Ronne 2005) and thus

experiments were limited to 10 days to avoid possible discrepancies in developmental stages in samples treated with variable irradiations.

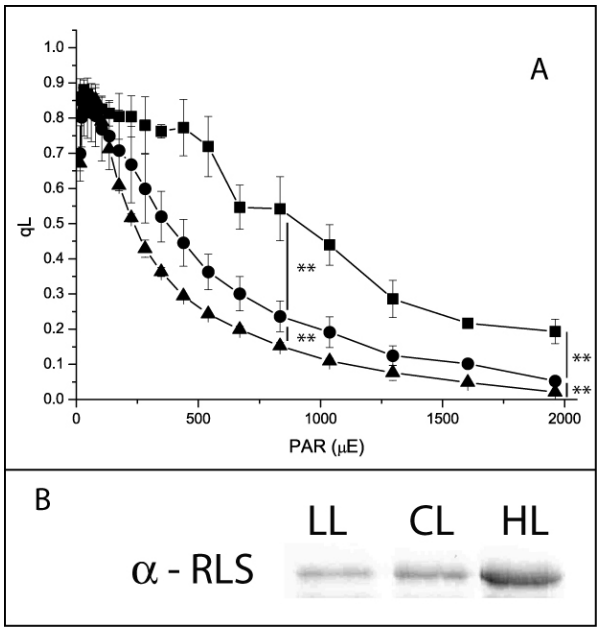

Figure 1. Modulation of photochemical activity in *P. patens* plants grown under different light conditions. A) Dependence of photochemical activity (indicated by the fluorescence parameter qL) from incident light in plants grown for five days in different light conditions. LL, CL and HL plants are shown in triangles, circles and squares respectively. SD (n = 3) is also reported. Asterisks indicate qL values are significantly different from the CL sample (t test, ** p =0.01). B) Western blotting against the RuBisCO large subunit (RLS). 1 μg of Chls of cellular extract were loaded for each sample. The antibody signal was verified to be linearly proportional to antigene amounts by loading different dilutions of the same sample.

First of all we tested all plants for their actual capacity of acclimating to different light conditions by measuring photochemical efficiency under different illuminations, estimated from the parameter qL (Kramer et al. 2004) . As shown in figure 1A, in LL plants qL decreases faster with the increasing light intensity, suggesting that here photosynthesis reaches saturation before control and high light treated plants. These data show that *P. patens* is indeed able to acclimate and modulate photosynthetic capacity in response to environmental conditions. This conclusion is consistent with the observed increase in RuBisCO, the key enzyme for CO_2 fixation (Figure 1B), as usually observed in HL acclimated plants (Gray et al. 1996;Hurry et al. 1994). We also checked if light intensity induced damages to the photosynthetic apparatus of our samples by measuring the PSII quantum yield (Fv/Fm) at the end of each light treatment. As reported in table 1, HL plants showed reduced Fv/Fm as compared to CL and LL. However, daily data during the light treatment show that damages are experienced in the first two days of illumination, while later plants reached a stationary state where there are no additional damages (Supplementary figure S1). Interestingly in the same

two days interval we also observe an increase in NPQ capacity (Supplementary figure S1).

We also evaluated changes in pigment content of acclimated plants and observed that Chl a/ b ratio, differently from what is normally observed in vascular plants, is not majorly affected by light intensities (Table 1). In HL plants we even observed a small decrease in Chl a/b, while the opposite was expected for a reduced antenna size. In LL plants Chl a/b ratio is identical to control conditions. This result suggests that photosynthetic antenna size in *Physcomitrella* does not change according to light conditions, in agreement with other observations in other mosses species (Koskimies-Soininen & Nyberg 1991;Rincon 1993). Instead, Car/Chl ratio is strongly increased in HL (Table 1), as commonly observed under strong illumination in vascular plants and mosses as well. When individual carotenoids are considered, we observed an increase in zeaxanthin and lutein content while violaxanthin is decreased. On the contrary neoxanthin remains stable, consistently with the suggested constant content of antenna proteins, where this carotenoid is bound specifically (Table 1).

	Fv/Fm	**Chl a / b**	**Car / 100 Chls**	**Neoxanthin /100 Chls**	**Lutein /100 Chls**
LL	0.83 ± 0.02	2.47 ± 0.10	23.5 ± 1.9	3.44 ± 0.40	11.46 ± 0.72
CL	0.81 ± 0.02	2.41 ± 0.13	22.8 ± 1.0	3.10 ± 0.64	11.77 ± 0.70
HL	0.73 ± 0.05*	2.22 ± 0.13*	33.5 ± 3.5**	3.72 ± 0.36	16.09 ± 1.93**

	Violaxanthin/ 100 Chls	**Anteraxanthin / 100 Chls**	**Zeaxanthin /100 Chls**	**β-Carotene /100 Chls**
LL	3.86 ± 0.31*	0.16 ± 0.02*	Nd	4.62 ± 1.39
CL	3.41 ± 0.25	0.44 ± 0.06	0.38 ± 0.08	3.71 ± 1.16
HL	1.42 ± 0.60**	1.72 ± 0.73**	5.52 ± 0.94**	5.06 ± 0.96

Table 1. PSII quantum yield and pigment content in *P. patens* WT plants grown under different light intensities. PSII quantum yield (Fv/Fm) was evaluated in plants grown in different light conditions (LL, CL, HL). In the same plants Chl a/b and Car/Chl ratios were also evaluated. Content in carotenoids of the xanthophyll cycle are also reported (expressed in mol / 100 Chls molecules). Values are reported as mean ± SD (n = 6). Asterisks indicate values significantly different from the CL sample (t test,* p = 0.05, ** p = 0.01). nd = non detected.

NPQ is enhanced in Physcomitrella plants acclimated to HL.

When grown in high light (HL), *P. patens* developed stronger NPQ as compared with both control (CL) and low light (LL) acclimated plants (Figure 2A). In all cases actinic light was set to 830 µE m^{-2} s^{-1} which we showed for CL plants to be sufficient to ensure maximal NPQ activation (Alboresi et al. 2010). Increasing the actinic light does not induce a stronger final NPQ neither in HL plants, as shown in figure 3.

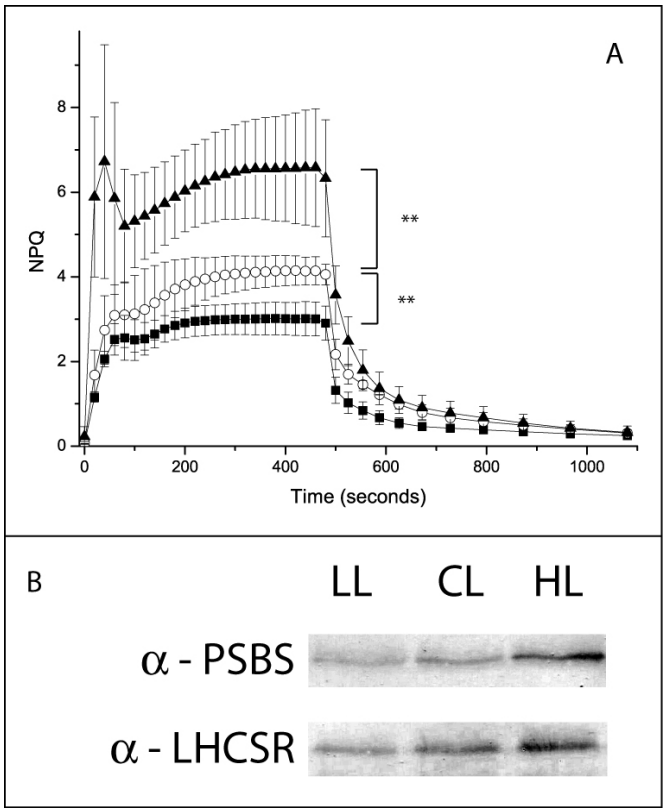

Figure 2. NPQ capacity of acclimated mosses. A) NPQ induction and relaxation of WT mosses acclimated to different light intensities. Data for HL, CL and LL plants are shown respectively in triangles, empty circles and squares. Bars indicate SD (n = 6). Actinic light (830 µE m^{-2} s^{-1}) was switched off after 8 minutes. Actinic light intensity during the measurement was chosen to be just sufficient to saturate photosynthesis in control plants and ensure maximal NPQ amplitude. Asterisks indicate when final NPQ values are significantly different from the CL sample (t test, ** p =0.01, n = 6).
B) Western blotting analyses of PSBS and LHCSR in thylakoids purified from differently acclimated plants. 4 and 1 Chl µg were loaded respectively. The antibody signal was verified to be linearly proportional to antigene amounts by loading different dilutions of the same sample.

In all cases NPQ had a fast activation and was completely relaxed upon switching off light, indicating that the strong NPQ observed is entirely due to its fastest component, known as qE or Feedback De-excitation (Kulheim, Agren, & Jansson 2002;Li et al. 2000). This result is consistent with the observation that NPQ is completely abolished by nigericin, an uncoupler which dissipates pH gradient across the thylakoid membrane and affects specifically qE activation (Supplementary Figure S2A).

The time course of NPQ in HL samples exhibited a complex shape with a fast raise followed by a partial relaxation and a new increase. This profile suggests a multiple components kinetic with a relaxation phase unexpected under the strong actinic illumination employed here. A similar behavior was previously observed in higher plants measured with low actinic light and was attributed to the activation of the Calvin-Benson cycle which induced an increase in ATP hydrolysis, ΔpH consumption and thus a NPQ relaxation (Cardol et al. 2010;Finazzi et al. 2004;Joliot & Finazzi 2010). A direct inspection of the fluorescence time curve (Supplementary Figure S2B) showed an increase of basal fluorescence F which confirms the hypothesis of a transient quenching relaxation. As a further confirmation that the underlying mechanism might be the same, we measured the NPQ in HL and CL acclimated samples of *P. patens* using different actinic light intensities.

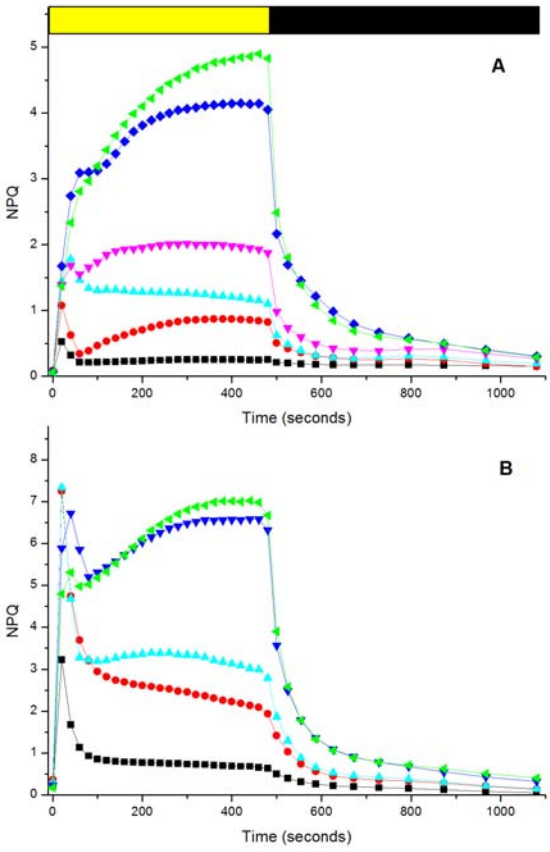

Figure 3. NPQ kinetics in HL and CL plants measured using different actinic light intensities. A) NPQ kinetics in CL acclimated plants measured at different actinic light intensities: 100, 200, 340, 540, 830 and 2000 µE m^{-2} s^{-1}, shown respectively in black, red, light blue, purple, blue and green. Actinic light was switched off after 8 minutes. B) NPQ kinetics measured in HL-acclimated plants. 100, 200, 340, 830, 2000 µE shown respectively in black, red, light blue, blue and green.

As shown in figure 3A, in CL samples we observed a behavior similar to vascular plants, with an NPQ relaxation detectable at lower actinic light intensity but not at stronger light. In the case of HL acclimated plants, instead, NPQ relaxation, although more

evident with dimmer actinic light, is still observable also with the strongest light treatment (Figure 3B). Thus, the transient relaxation of quenching during NPQ induction can be attributed to an induction of metabolism driving to reducing equivalents consumption. This metabolic activity is detectable in HL grown mosses by an increase in fluorescence at all actinic light intensities, differently from what is generally observed in vascular plants.

Role of PSBS and LHCSR in light acclimation: analysis of KO mutants

As mentioned previously, NPQ in *P. patens* depends on the presence of both PSBS and LHCSR (Alboresi et al. 2010). In order to test whether one of these proteins was preferentially accumulated during acclimation, we evaluated their content upon long-term treatment. As shown in figure 2B, following HL acclimation in WT samples we observed over-expression of both PSBS and LHCSR, suggesting that both proteins are actually involved in the NPQ up-regulation observed in HL. In order to better understand the role of these protein components on acclimation and NPQ modulation, we grew knock-out (KO) mutants depleted in either PSBS and/or LHCSR under different light regimes. We employed five mutant lines, three single mutants respectively depleted in PSBS (*psbs* KO) and in each of the two copies of LHCSR present in the *Physcomitrella* genome (*lhcsr1* KO) and (*lhcsr2* KO), the double mutant depleted in both PSBS and LHCSR1 (*psbs lhcsr1* KO) and the triple mutant (*psbs lhcsr1 lhcsr2* KO) lacking all three genes. Among the two LHCSR copies, LHCSR1 encodes for the largest fraction of the protein and its deletion causes the strongest NPQ phenotype, while *lhcsr2* KO is more similar to WT (Alboresi et al. 2010). Preliminary analyses on *lhcsr2* KO showed very small differences with WT even when grown in HL and we thus focus here on *lhcsr1* KO only. Moreover, clearer information on the role of LHCSR2 can be obtained from the phenotype of *psbs lhcsr1* KO double mutant, where LHCSR2 is the only responsible for the residual NPQ (Alboresi et al. 2010).

All mutants tested showed no major alteration of their photochemical efficiency in control conditions, as estimated by their Fv/Fm values (Alboresi et al. 2010). We also checked that mutation did not affect the capacity of generating a transmembrane potential by measuring the carotenoid electro-chromic shift (ECS, (Bailleul et al. 2010;Baker, Harbinson, & Kramer 2007)). As shown in supplementary figure S3, light induced ECS is equivalent in WT and the triple *psbs lhcsr1 lhcsr2* KO. This verification is important also because ΔpH is a key signal for activation of other protection mechanisms over NPQ, like the xanthophyll cycle (Arnoux et al. 2009).

Despite this ability to induce ΔpH as well as the WT, some mutants showed PSII photoinhibition when grown in HL, as evidenced by a strong decrease in PSII

photosynthetic efficiency (Table 2). In particular, the triple *psbs lhcsr1 lhcsr2* KO mutant showed the strongest photoinhibition as compared to WT mosses grown in the same conditions. Interestingly, this mutant is also unable to activate any NPQ in all light conditions tested (Figure 4D), suggesting NPQ was effective in preventing the light-induced damage under prolonged illumination.

Fv/Fm	**WT**	***lhcsr1* KO**	***psbs* KO**	***psbs lhcsr1* KO**	***psbs lhcsr1 lhcsr2* KO**
LL	0.83 ± 0.02	0.83 ± 0.03	0.81 ± 0.07	0.84 ± 0.04	0.83 ± 0.02
CL	0.81 ± 0.02	0.82 ± 0.03	0.81 ± 0.03	0.80 ± 0.01	0.82 ±0.02
HL	0.73 ± 0.05*	0.72 ± 0.04*	0.72 ± 0.08*	0.70 ± 0.03**	0.54 ± 0.07** ##
Car / 100 Chls in HL	33.5 ± 3.5	33.8 ± 2.4	34.4 ± 3.6	32.5 ± 1.2	43.6 ± 1.6 ##

Table 2. PSII quantum yield and carotenoid content in *P. patens* WT and NPQ mutants grown in different light conditions. PSII quantum yield (Fv/Fm) was evaluated in *P. patens* plants grown in different light conditions (LL, CL, HL). Values are reported as mean ± SD (n = 6). Asterisks indicate values significantly different from the CL sample of the same genotype (t test, * p = 0.05, ** p =0.01), while # indicates samples different from WT grown in the same conditions (t test, ## p =0.01, n = 6). Total carotenoid content of HL acclimated plants is also reported. # indicates samples significantly different from WT grown in the same conditions (t test, ## p =0.01, n = 6)

Results reported in figure 4 show that single KO mutants depleted in PSBS (*psbs* KO, Figure 4A) or LHCSR1 isoform (*lhcsr1* KO, Figure 4B) increased their NPQ capacity in HL, implying that both LHCSR and PSBS are involved in NPQ enhancement upon HL acclimation. Instead, NPQ is not increased in the double mutant *psbs lhcsr1* KO (Figure 4C). Since LHCSR2 has been shown to be active in quenching in control conditions (Alboresi et al. 2010), this finding suggests that LHCSR2 level is not significantly increased upon HL acclimation. Western blot analysis (Figure 5) on light-acclimated mosses confirms that both LHCSR and PSBS are over-expressed in HL. In fact, *psbs* KO and *lhcsr1* KO over-express respectively LHCSR and PSBS proteins, indicating that the expression of PSBS and LHCSR are not mutually regulated but largely independent from each other and that the expression of both proteins is regulated by a stress stimulus. In *lhcsr1* KO and in the double mutant *psbs lhcsr1* KO, the band detected by LHCSR antibody is only due to LHCSR2. In these genotypes, no alterations in LHCSR2 protein accumulation was observed upon acclimation to different conditions, confirming the conclusion driven from NPQ kinetics that LHCSR2 is not induced by HL.

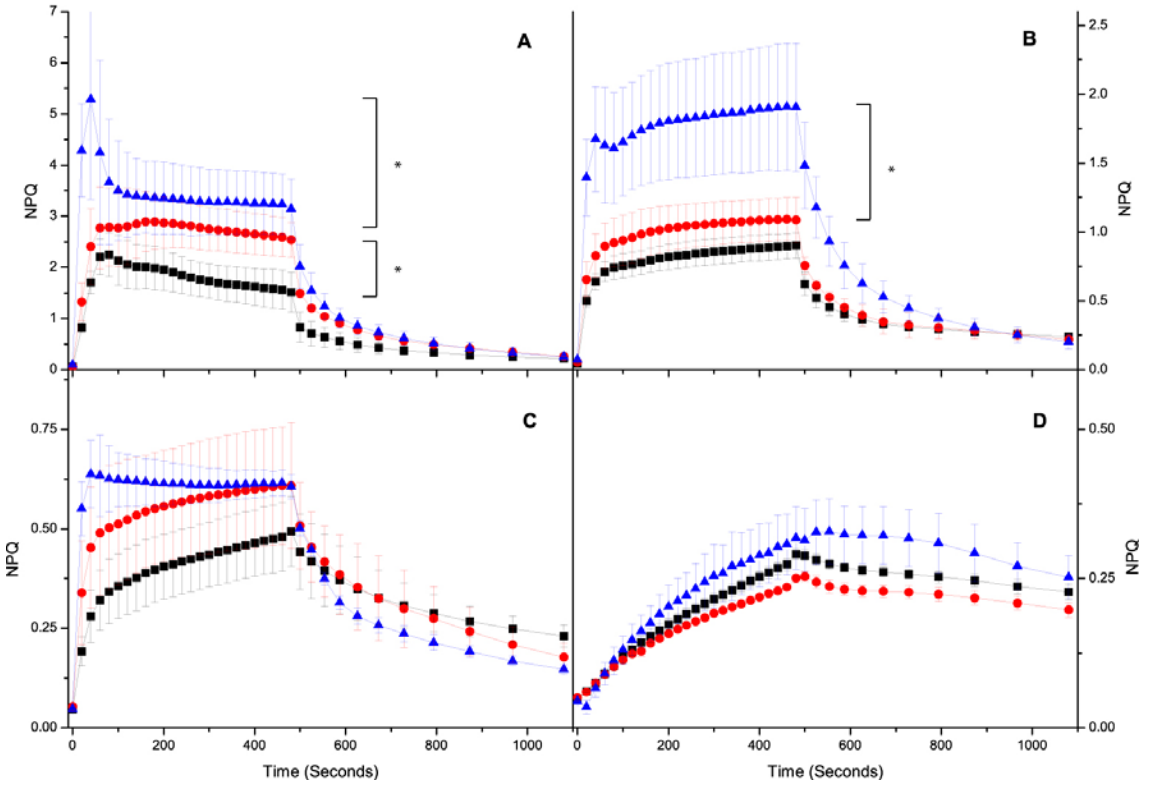

Figure 4. NPQ response in HL acclimated mutants. NPQ induction and relaxation of mosses acclimated to different light intensities. Data for HL, CL and LL plants are shown respectively in blue triangles, red circles and black squares. Bars indicate SD (n = 4). Actinic light (830 µE m^{-2} s^{-1}) was switched off after 8 minutes. Results from *psbs* KO, *lhcsr1* KO, *psbs lhcsr1* KO and *psbs lhcsr1 lhcsr2* KO are shown respectively in A, B, C and D. Asterisks indicates when final NPQ values are significantly different from the CL sample (t test, * p = 0.05, n = 4). In the case of *psbs* KO significance was calculated for maximal NPQ. Note that in different panels the Y axis scale is different, decreasing from A to D.

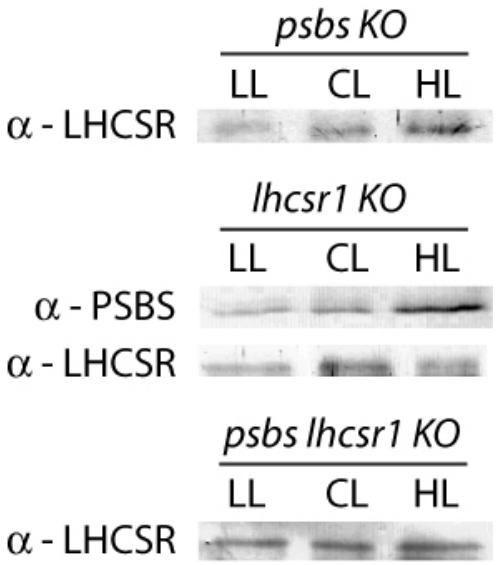

Figure 5. Western Blotting on light acclimated mutants. Western blotting analysis of LHCSR and PSBS in thylakoids purified from mutants acclimated to different light conditions. 4 Chl µg were loaded per lane for blots against PSBS. In the case of western against LHCSR, we loaded 3 µg in *lhcsr1* KO and *psbs lhcsr1* KO and 1 µg in *psbs* KO. To verify signal linearity with protein amount in the same gel different sample loads were analyzed. We also tested that KO phenotype was retained and we observe no expression of the depleted protein/proteins in the mutants, as expected.

Significant differences in NPQ time course may also be observed for the different mutants. In HL acclimated *psbs* KO mosses, the transient NPQ relaxation described above for the WT is more evident, suggesting a correlation with the presence of LHCSR. However, it should be pointed out that NPQ transient relaxation is also visible in genotypes lacking LHCSR, especially when lower actinic light intensities are applied (not shown). The most likely explanation is that NPQ transient relaxation is independent from both PSBS- and LHCSR-dependent mechanisms, but it is more easily observed in the presence of the strong LHCSR-dependent NPQ.

We also analyzed the pigment composition of the acclimated mutants. It is worth noting that all genotypes, independent from growth conditions, show similar Chl a/b ratio, suggesting no major modifications in Chl binding proteins nor alterations in antenna size with respect to WT under different illumination. Interestingly, while most mutants showed carotenoids accumulation in HL similar to WT, this was significantly higher in the triple KO mutant, confirming an increased stress experienced by this mutant (Table 2). We also analyzed the content in different xanthophyll species, finding in all HL acclimated samples an increased level of zeaxanthin and lutein as observed in WT (Table 1).

Acclimation to low temperature

Low temperatures are well known to enhance photoinhibition and cold response in part overlaps with the one induced by high light (Huner et al. 1998). In order to verify if low temperature, alike high light, was effective in inducing a modulation of NPQ, *P. patens* was grown at low temperature (4°C) while maintaining the same light intensity as in control conditions. The analysis of Fv/Fm index shows that WT samples are able to acclimate to these conditions and only specific mutants showed a small reduction of photochemical efficiency when grown at dim light (Table 3). The stronger decrease in photosynthetic efficiency was observed in those mutants where NPQ capacity was most affected (*lhcsr1* KO, *psbs lhcsr1* KO and triple *psbs lhcsr1 lhcsr2* KO mutant), suggesting that NPQ might play a role also in acclimation to low temperature.

	WT	***lhcsr1* KO**	***psbs* KO**	***psbs lhcsr1* KO**	***psbs lhcsr1 lhcsr2* KO**
24°C, CL	0.81 ± 0.02	0.82 ± 0.03	0.81 ± 0.03	0.80 ± 0.01	0.82 ± 0.02
4°C, CL	0.80 ± 0.03	0.75 ± 0.05*	0.79 ± 0.04	0.75 ± 0.04*#	0.71 ± 0.02**##

Table 3. PSII quantum yield in *P. patens* WT and NPQ mutants grown at different temperatures. PSII quantum yield (Fv/Fm) was evaluated in *P. patens* plants grown at 24 and 4°C, always with CL illumination (45 µE m^{-2} s^{-1}). Values are reported as mean ± SD (n = 6). Asterisks indicate values significantly different from the 24°C sample (t test, * p = 0.05, ** p =0.01), while # indicates samples significantly different from WT grown in the same conditions (t test, # p=0.05, ## p =0.01, n = 6).

As shown in figure 6, samples of WT *P. patens* grown in the cold showed increased NPQ with respect to control conditions. NPQ time course was also characterized by a fast rise followed by a partial relaxation, similar to what observed in HL conditions (Figure 6A). When different KO mutants where grown in the same conditions, we observed an increase of NPQ in *lhcsr1* KO mutant (Figure 6C) and NPQ maximal value also increased in *psbs* KO (Figure 6B), suggesting that both PSBS and LHCSR are involved in NPQ enhancement upon cold acclimation, similarly to what was observed in high light, although the latter seems to be less influenced by low temperature. In cold acclimated samples we observed a significant induction of NPQ including the *psbs lhcsr1* KO double mutant (Figure 6D), which is instead in contrast with the case of HL acclimation. In this genotype NPQ only depends on LHCSR2 and thus this observation suggests that a specific accumulation of the LHCSR2 protein is induced by low temperature. This hypothesis is also consistent with the observation that the *lhcsr2* KO showed reduced NPQ with respect to WT (Supplementary Figure S4A). Instead, as in HL, no NPQ induction by cold acclimation was observed for the triple KO mutant (Supplementary Figure S4B). Western blotting analysis using specific antibodies confirmed the conclusions from NPQ kinetics, with a PSBS accumulation particularly evident (Figure 6E). Chl a/b ratio in cold-acclimated mosses was unchanged while Car/Chl ratio increased, alike in HL acclimation (Supplementary Figure S5).

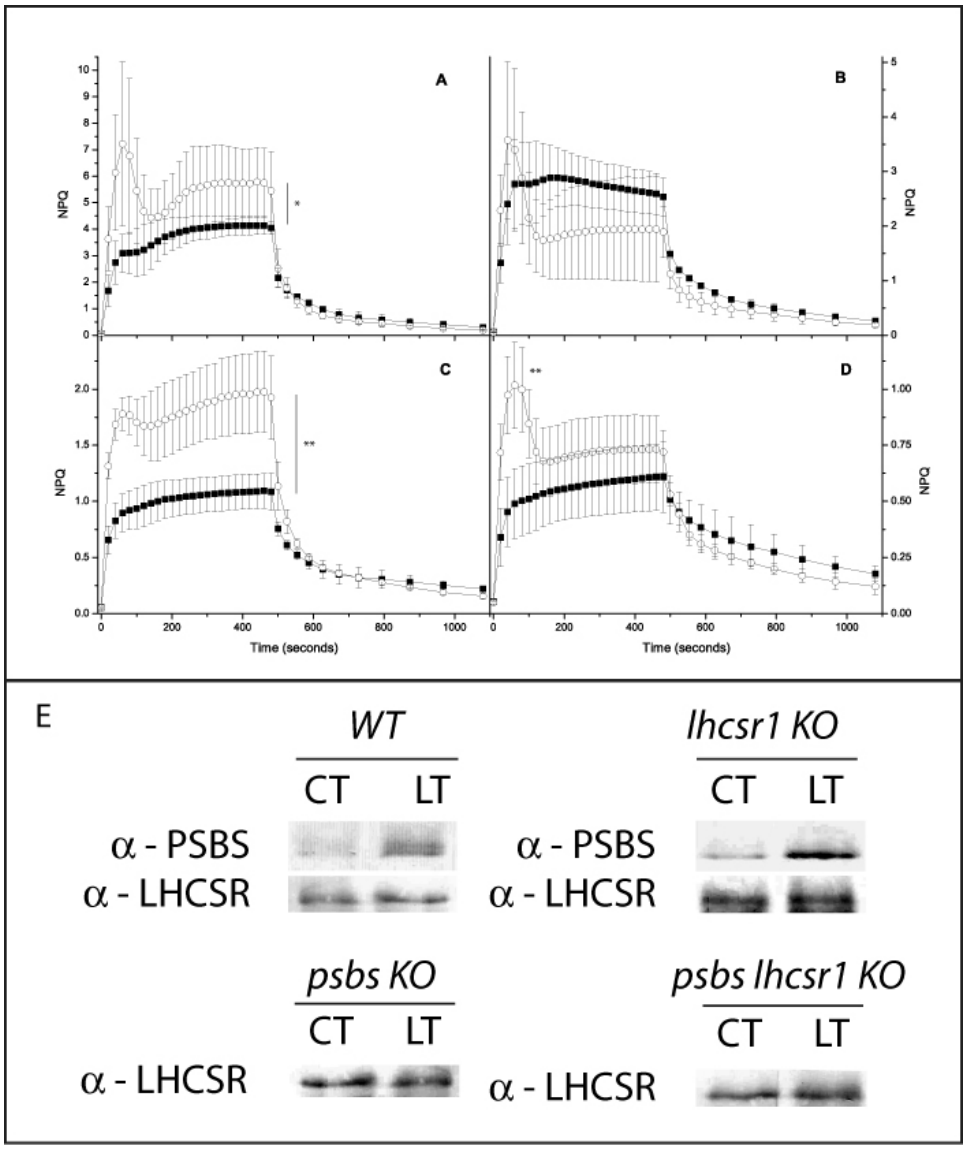

Figure 6. NPQ induction and Western Blotting of WT and mutants grown at low temperature. A-D) NPQ induction and relaxation of mosses acclimated to room and low temperatures are compared. Data for plants grown at 24 and 4°C are shown respectively in black squared and open circles. Bars indicate SD (n = 4). Actinic light (830 µE m^{-2} s^{-1}) was switched off after 8 minutes. WT, *psbs* KO, *lhcsr1* KO, *psbs lhcsr1* KO are shown respectively in A, B, C and D. Note that in different panels scales are different, decreasing from A to D. *lhcsr2* KO and *psbs lhcsr1 lhcsr2* KO are shown in supplementary figure S4. Asterisks indicate when NPQ values are significantly different from the 24°C sample (t test, * p = 0.05, ** p =0.01). In WT and *lhcsr1* KO this was calculated for final NPQ while in *psbs lhcsr1* KO for the maximal NPQ. E) Western blotting analysis of LHCSR and PSBS in thylakoid purified from mutants acclimated to different light conditions. 4 Chl µg were loaded for blots against PSBS while for LHCSR we loaded 3 µg in *lhcsr1* and *psbs lhcsr1* KO and 1 µg in WT and *psbs* KO. To verify signal linearity with protein amount in the same gel also other protein amounts were loaded. CT and LT indicate respectively control and low temperature samples.

DISCUSSION

PSBS and LHCSR are responsible for NPQ modulation during acclimation of P. patens.

In this work we analyzed the effect of growing *Physcomitrella* plants in different light and temperature conditions on NPQ amplitude and kinetics. We observed that HL grown plants have a stronger and faster NPQ with respect to control plants, which was correlated with the increased accumulation of PSBS and LHCSR, the proteins responsible for NPQ activity in *P. patens* (Alboresi et al. 2010) (Figure 2). The analysis of mutants depleted in PSBS and/or LHCSR acclimated to the same conditions showed that both proteins are involved in the modulation of NPQ response. Thus, *Physcomitrella* plants are able to modulate Non Photochemical Quenching levels by regulating the concentration of PSBS and/or LHCSR. A similar picture was observed in the case of *Chlamydomonas reinhardtii*, where the increase in LHCSR content was responsible for the stronger NPQ observed in HL treated cells (Peers et al. 2009). Also in the case of *Arabidopsis*, over-expressing plants demonstrated that modulation of PSBS accumulation affects NPQ intensity (Li et al. 2002;Li, Gilmore, & Niyogi 2002).

We further observed that plants depleted in PSBS and LHCSR not only showed a reduced NPQ capacity but also a larger reduction of PSII photochemical yield, a clear indication of photoinhibition. This result suggests that the capacity of modulating NPQ is important for mosses capacity of responding to intense illumination so as to avoid radiation damages. This finding is particularly relevant if we consider that NPQ is known to be a fast activated mechanism which, in *Arabidopsis*, was suggested to play a major role in rapid changes of light intensity and to be less relevant for photoprotection of plants exposed to intense but constant illumination (Kulheim et al. 2002).

LHCSR1 and LHCSR2 isoforms are differentially regulated.

P. patens genome has two sequences encoding LHCSR proteins with LHCSR1 being accumulated at higher levels and responsible for most NPQ activity (Alboresi et al. 2010). The two sequences have 91% aminoacid identity, suggesting a similar biochemical activity. Consistent with this idea is the observation that LHCSR2 is capable of activating NPQ as demonstrated by the comparison of *psbs lhcsr1* KO and the triple *psbs lhcsr1 lhcsr2* KO (Alboresi et al. 2010).

Here, we further report that LHCSR1 accumulation is induced under strong illumination as shown by western blotting and NPQ kinetics. On the contrary, *psbs lhcsr1*KO mutants, which rely only on LHCSR2 for their NPQ activation, did not show any significant increase in NPQ or in LHCSR accumulation when grown in HL, suggesting that this isoform is not light-induced (Figures 4 and 5).

On the contrary, LHCSR2 content responded to the growth in low temperature under dim illumination. In fact, both LHCSR protein level and NPQ amplitude are increased in *psbs lhcsr1* KO grown at 4°C with respect to control samples (Figure 6). LHCSR1 accumulation is instead less influenced by temperature, suggesting that the two functional *lhcsr* genes are differentially regulated by environmental factors. These data suggest the presence of two LHCSR isoforms with different regulation which might provide optimal photoprotection to *P. patens* plants in different growth conditions.

Specificity of photosynthetic acclimation in mosses.

The capacity of mosses to respond to changing light conditions is here clearly demonstrated by the increase in their photochemical capacity and RuBisCO content, similarly to what was previously observed in other plants and algal species grown under different illumination (Bailey et al. 2004;Ballottari et al. 2007;Walters 2005). Vascular plants exposed to different light intensities also show a change in Chl a/b ratio mainly due to the modulation of the size of their PSII antenna system. On the contrary, we observed that Chl a/b ratio remained substantially stable in all different growing conditions in *P. patens*, suggesting that antenna size of Photosystems is instead maintained unchanged. It is worth underlining that HL grown *P. patens* plants displayed a clear increase in Car/Chl ratio, a response to strong illumination normally occurring in parallel with changes in Chl a/b ratio in tracheophyta. This result suggests that the observed stability of Chl a/b ratio is not due to an insufficient strong light treatment but, rather, is a specific characteristic of *P. patens*. Consistently with this hypothesis, previous reports from other moss species also showed a constant Chl a/b ratio upon growth under different light intensities (Koskimies-Soininen et al. 1991;Rincon 1993). Taken together, these data suggest that at least some mosses, differently from vascular plants, do not reduce the size of their antenna system in response to strong illumination.

A possible explanation for this behavior can be proposed considering that bryophytes are poikilohydric organisms and thus their hydration state is mainly determined by the environment. Under intense illumination mosses experience desiccation with the block of photosynthetic activity and the activation of a strong thermal dissipation mechanism dissipating all absorbed energy (Heber 2008;Heber, Lange, & Shuvalov 2006). Thus, we may hypothesize that mosses, different from vascular plants, do not need to optimize their photosynthetic apparatus for operating under strong illumination since in high light they are not photosynthetically active. On the contrary, they must operate with the maximal efficiency when (e.g. dawn) or where (e.g. under dense canopies) their tissues are hydrated and photosynthetically active. Normally in these high humidity conditions incident light is also low and thus the maintenance of a large antenna is likely to provide

an advantage in terms of light harvesting efficiency. Differently from vascular plants such a large antenna is not a disadvantage under strong light because tissues are rapidly dehydrated and photosynthesis is turned off.

This picture where mosses always maintain a large antenna system can also provide a possible explanation for the presence of such an intense NPQ in *P. patens:* since mechanisms of long-term modulation of the light harvesting apparatus are reduced, a highly efficient NPQ may be functional in case of short-term light stress. In fact mosses, even under a canopy, can be exposed to light excess in the case of sunflecks which might saturate photosynthesis and induce oxidative stress. In this case the capacity of inducing a strong NPQ provides an efficient protection and is possibly for this reason that these species retained both LHCSR- and PSBS-dependent NPQ mechanism, respectively typical of green algae and vascular plants. In addition, this strong NPQ capacity, besides being a fast activated process, seems to ensure an efficient protection of the low-light adapted photosynthetic apparatus in *P. patens* even in the case of continuous long-term light stress.

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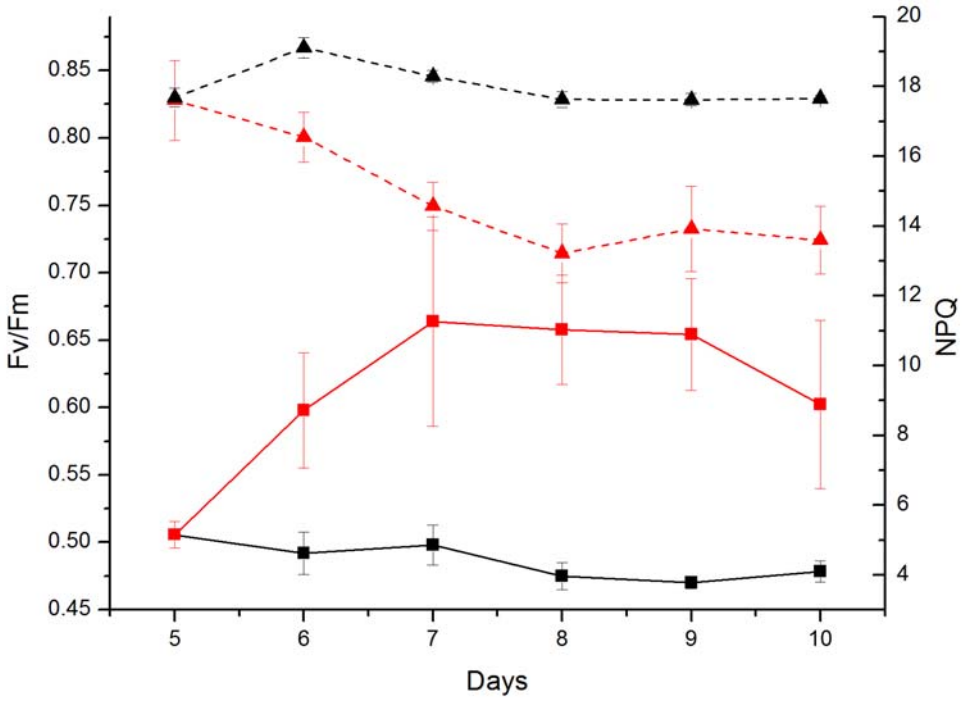
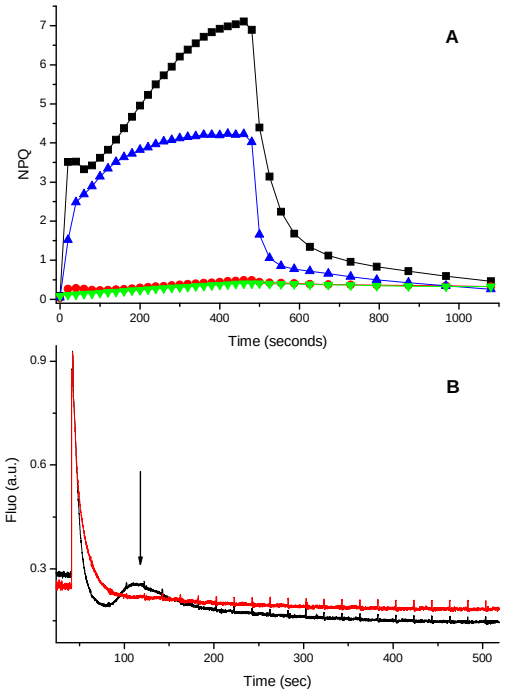
SUPPORTING INFORMATION

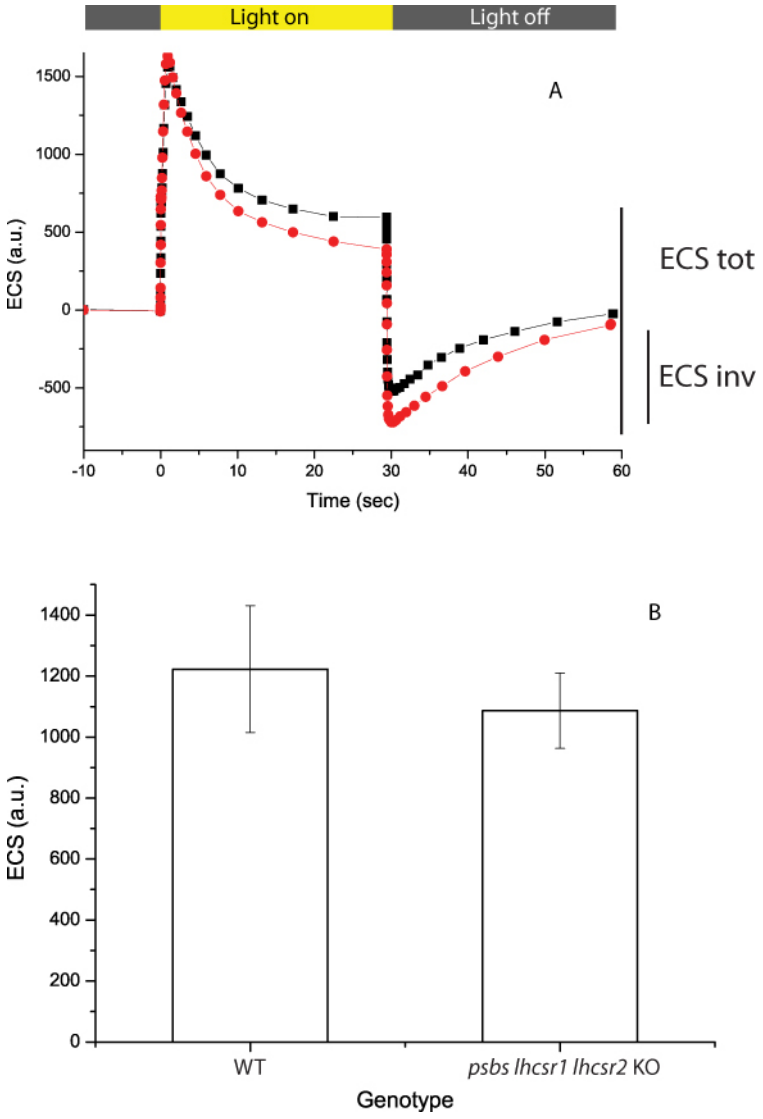
Supplementary Figure S1. PSII quantum yield and NPQ capacity during WT plants acclimation to HL . Photosystem II quantum yield evaluated from Fv/Fm (circles) was followed for WT plants grown in CL (black) and HL (red). In the same plants also NPQ was monitored and values measured after 8 minutes of light treatment are shown in black and red squares respectively for CL and HL plants.



Supplementary Figure S2. NPQ dependence from ΔpH in *P. patens* plants. A) NPQ induction in plants is completely due to its fastest component qE, as shown by the dependence from ΔpH. In the presence of nigericine (red) the strong NPQ observed in HL samples (black, with only 150 mM sorbitol) is completely abolished. The same is observed for plants grown in CL, shown in blue (control) and green (nigericin treated). B) Fluorescence kinetics. Values are normalized to the Fm value. HL and CL plants are shown in black and red respectively, measured with 830 µE of actinic light.



Supplementary Figure S3. Evaluation of light induced transmembrane potential across the thylakoids membranes in WT and *P. patens* mutants. Transmembrane potential was estimated from carotenoid ECS. Light adapted WT and the triple *psbs lhcsr1 lhcsr2* KO plants are shown. A) ECS signal was measured for 30 seconds of illumination at 940 µE m^{-2} s^{-1} and then for additional 30 seconds in the dark for WT (Black squares) and triple *psbs lhcsr1 lhcsr2* KO mutant (red circles). When light is switched off the decrease in ECS represent the total transmembrane potential (ECS tot) while the following recovery (ECS inv) allows estimating ΔpH component. Data were normalized to the Fmax measured in the same sample after 20 minutes of dark adaptation in order to compare the same amount of Chls under illumination. B) Total ECS values, after normalized to maximal fluorescence in WT and triple *psbs lhcsr1 lhcsr2* KO. Data are the average of at least 4 repeated measure on 3 independent samples.



Supplementary Figure S4. NPQ induction in *P. patens* mutants grown at low temperature. NPQ induction and relaxation of mosses acclimated to room and low temperatures are compared. Data for plants grown at 24 and 4°C are shown respectively in black squares and red circles. Bars indicate SD (n = 4). Actinic light (830 µE m^{-2} s^{-1}) was switched off after 8 minutes. *lhcsr2* and *psbs lhcsr1 lhcsr2* KO are shown in A and B respectively. Note that in different panels have different Y axis values.

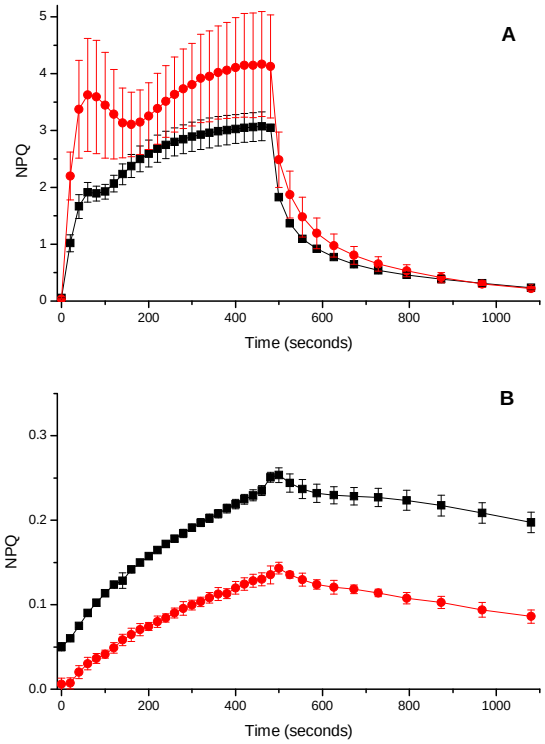


Figure S5. Pigment composition in cold acclimated plants. Chl a/b and Car/Chl ratio were measured in *P. patens* plants grown under different temperatures with the same illumination (CL, 45 µE m^{-2} s^{-1}). Content in carotenoids of the xanthophyll cycle are also reported (expressed in mol / 100 Chls molecules). Values are reported as mean ± SD (n = 4). Asterisks indicate values significantly different from the 24°C sample (t test,* ** p = 0.01, n= 4).

	Chl a / b	**Car / 100 Chls**	**Violaxanthin/ 100 Chls**	**Anteraxanthin / 100 Chls**
24°C, CL	2.41 ± 0.13	22.8 ± 1.0	3.41 ± 0.25	0.44 ± 0.06
4°C, CL	2.45 ± 0.10	36.9 ± 5.4**	1.45 ± 0.10**	2.37 ± 0.16**
	Zeaxanthin /100 Chls	**Neoxanthin /100 Chls**	**Lutein /100 Chls**	**β-Carotene /100 Chls**
24°C, CL	0.38 ± 0.08	3.10 ± 0.64	11.77 ± 0.70	3.71 ± 1.16
4°C, CL	4.67 ± 0.16**	3.02 ± 0.76	15.58 ± 0.50**	6.48 ± 2.17

CHAPTER 4

TWO INDEPENDENT MECHANISMS FOR NPQ FROM PLANTS AND ALGAE OVERLAPPED DURING LAND COLONIZATION

TWO INDEPENDENT MECHANISMS FOR NPQ FROM PLANTS AND ALGAE OVERLAPPED DURING LAND COLONIZATION

ABSTRACT

Light is the source of energy for photosynthetic organisms but, when in excess, it drives the formation of reactive oxygen species and photoinhibition. Plants and algae avoid oxidative damage by activating Non Photochemical Quenching (NPQ), which consists in the rapid dissipation of excess excitation energy as heat. Although NPQ is found in both groups of organisms, algae and plants rely on two different proteins for its activation, known as LHCSR and PSBS, respectively. In the moss *Physcomitrella patens* both these proteins are present and active in inducing NPQ. We here analyzed *P. patens* plants depleted or over-expressing PSBS and/or LHCSR and we showed that PSBS and LHCSR are capable of activating NPQ independently the one from the other, suggesting a different and autonomous activation mechanisms. These results suggest a picture for the evolution of photoprotection mechanisms during land colonization where PSBS dependent mechanism took the place of the one dependent on LHCSR present in algae after a significant time of overlapping.

INTRODUCTION

In the introduction and in the previous chapters 2 and 3 we extensively discussed how photosynthetic organisms have to face with a variable environment, where light can be harvested in excess with respect to the maximal photosynthetic capacity. Land colonization required adaptation of regulatory mechanisms since plants have a sessile form which prevents escaping rapid changes in light intensities by swimming away (Waters, 2003). Also the terrestrial environment is characterized by harsher condition as cycles of flooding/dessication, extremes in temperatures, increased exposure to UV radiations and light, higher concentration of oxygen and lower availability of carbon dioxide compared to water ecosystems (Waters, 2003; Becker & Marin, 2009). In these conditions, thus, it is more likely that energy is harvested in excess. During evolution, therefore, photosynthetic organisms had to adapt their photoprotection mechanisms to new conditions. We previously detailed discussed that the fastest protection mechanism is called Non Photochemical Quenching (NPQ) and consists in the thermal dissipation of the excited chlorophyll singlet states (^{1}Chl*) (Niyogi, 2000). NPQ is characterized by two major components, named qE (Energy Quenching) and qI (Inhibitory Quenching) (Niyogi,

2000; Szabo *et al.*, 2005). Recent data highlight that the fastest NPQ component, called qE (Energy quenching) relies on different proteins for its activation in algae and vascular plants, LHCSR in the former (Koziol *et al.*, 2007; Peers *et al.*, 2009; Zhu & Green, 2010; Engelken *et al.*, 2010; Bailleul *et al.*, 2010), while PSBS in the latter (Li *et al.*, 2000). Moreover, recent results from literature suggested that PSBS- and LHCSR-dependent NPQ mechanisms are likely different (Bonente *et al.*, 2008; Betterle *et al.*, 2009; Kereiche *et al.*, 2010) (Peers *et al.*, 2009; Bonente *et al.*, 2011), as detailed explained in the introduction. Both proteins, however, are similarly activated by the accumulation of protons in the lumen (Li *et al.*, 2002b; Bonente *et al.*, 2011).

Thanks to the generation of specific knock-out (KO) mutants depleted in PSBS and/or LHCSR, we recently demonstrated that both proteins are active in inducing NPQ in the moss *Physcomitrella patens* (Alboresi *et al.*, 2010), and that this process is of key importance to avoid damage of the photosynthetic apparatus also in prolonged excess light exposure (Chapter 3).

We here analyzed if in *Physcomitrella* plants LHCSR and PSBS are capable alone of activating NPQ or if they interact somehow. To this aim we again exploited *Physcomitrella* ability of homologous recombination to combine multiple KO mutations and to over-express (OE) PSBS or LHCSR proteins. These mutant lines showed that LHCSR and PSBS are active simultaneously and independently in *P. patens* and that they not interact, thus strongly supporting the hypothesis that they rely on a different molecular mechanism.

MATERIALS AND METHODS

Plant material. Protonemal tissue of *P. patens*, Gransden wild-type strain, *psbs* and/or *lhcsr* KO lines (Alboresi *et al.*, 2010) and PSBS or LHCSR over-expressor (OE) lines were grown on rich $PpNH_4$ or minimum $PpNO_3$ media (Asthon *et al.*, 1979) 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, PRESTON, UK) as previously described (Alboresi *et al.*, 2008). 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^{-2} s^{-1}.

Protoplast transformation and KO and OE generation. Plasmids for targeted KO generation for PSBS or LHCSR1/2 polypeptides were already obtained in (Alboresi *et al.*, 2010). In this work the same constructs and the single KO lines previously generated

(Alboresi *et al.*, 2010) were used to obtain double KO lines (KO transformations were mainly performed by Dr. Alessandro Alboresi, University of Verona). For the generation of OE mosses we used as starting material cDNA obtained from *P. patens* protonema grown in control conditions. Coding sequence for PSBS (XM_001778511.1) and LHCSR1 (XM_001776900.1) were amplified by PCR and successively cloned into pMAK1 vector (available at NIBB PHYSCObase (http://moss.nibb.ac.jp); kindly provided by Prof. Takashi Murata, National Institute for Basic Biology, Okazaki, Japan) using XhoI/HpaI and XhoI/ApaI restriction enzymes, respectively. LHCSR1 and LHCSR2 isoforms present 91% sequence identity, and thus we chose to over express LHCSR1 as it is the isoform strongly accumulated in *Physcomitrella* based on our previous results (Alboresi *et al.*, 2010). To obtain all the mutant lines presented in this work, *P. patens* transformation was performed as in (Schaefer & Zryd, 1997) with minor modifications, as we did in (Alboresi *et al.*, 2010) (chapter 2).

NPQ measurements. In vivo chlorophyll fluorescence of *P. patens* grown 10 days in minimum medium was measured at room temperature with Dual PAM-100 fluorometer (Walz), using a saturating light at 6000 µE m^{-2} s^{-1} and actinic light of 830 µE m^{-2} s^{-1}. Before measurements, plates were dark-adapted for 40 minutes at room temperature, as in chapters 2 and 3. The parameters Fv/Fm and NPQ were calculated as (Fm-Fo)/Fm, and (Fm-Fm')/Fm' (Demmig-Adams *et al.*, 1996). Data are presented as mean ± SD of 3 independent experiments.

Thylakoids extraction, pigments content, SDS-PAGE and Western Blotting analyses. Thylakoids from protonemal tissue (10 days old plants grown in minimum $PpNO_3$) were prepared using an *Arabidopsis* protocol with minor modifications (Alboresi *et al.*, 2008). Chl a/b and Chl/Car ratios were obtained by fitting the spectrum of acetone 80% pigment extracts with spectra of the individual purified pigments as in (Croce *et al.*, 2002). For immunoblotting analysis, following SDS-PAGE, proteins were transferred onto a nitro-cellulose membrane (Pall Corporation) using a blot system from Amersham and detected with specific home-made polyclonal antibodies.

RESULTS

Generation and characterization of double KO mutants for PSBS and/or LCHSR: all proteins are active alone in NPQ .

We previously showed that PSBS, LHCSR1 and LHCSR2 isoforms are active in *Physcomitrella* NPQ, since plants depleted in each of these proteins showed reduced NPQ (Alboresi *et al.*, 2010). In order to assess if they are active independently or interact somehow, we combined different KO mutations generating plants which retain only one of these subunits. The double mutants *psbs lhcsr1* KO, *psbs lhcsr2* KO and *lhcsr1 lhcsr2* KO, in fact, retain only one of the protein responsible for the NPQ, respectively LHCSR2, LHCSR1 and PSBS. While *psbs lhcsr1* KO was already described in (Alboresi *et al.*, 2010), we here generated the others, using the single *lhcsr2* KO and *lhcsr1* KO lines as background to obtain respectively *psbs lhcsr2* KO and *lhcsr1 lhcsr2* KO. Constructs are designed to substitute the entire coding sequences with selection cassettes, exploiting the *Physcomitrella* capacity of doing homologous recombination. Transformation led to the isolation of several independent stably resistant colonies, which were characterized by PCR to verify the presence of the insertion at the expected target site and select single copy insertions as described in more detail in (Alboresi *et al.*, 2010) (supplementary figure 1). 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 (supplementary figure 1). As in our previous work, all the lines retained for each transformation present an indistinguishable phenotype and thus we present data from one line for each mutation. The accumulation of PSBS/LHCSR proteins in the KO lines was analyzed by Western blotting (Figure 1A). As expected, we identified only PSBS in *lhcsr1 lhcsr2* KO plants while we detected only LHCSR in *psbs lhcsr2* KO. In the case of *psbs lhcsr1* KO, as already shown in (Alboresi *et al.*, 2010), LHCSR2 was not detectable without increasing the protein loading.

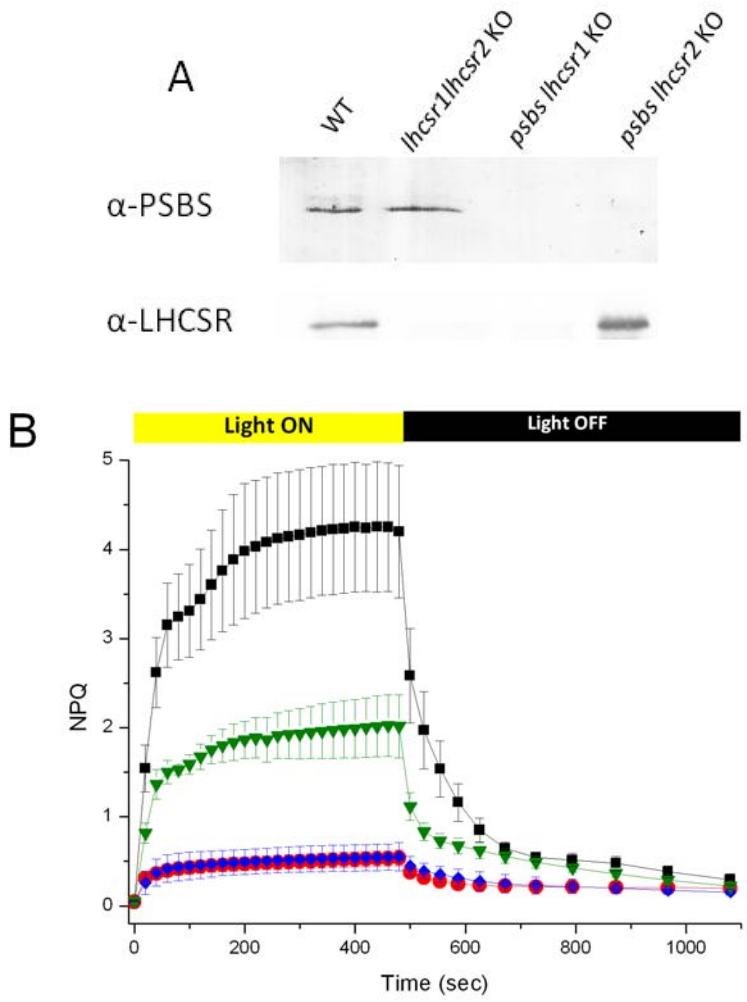


Figure 1. Western blot and NPQ phenotype analyses of double KO mutants. A) Western blotting using antibodies against PSBS and LCHSR polypeptides in thylakoids extracts of the selected lines. For blot against PSBS, 4 µg of Chl were loaded for each sample, while for anti-LHCSR we loaded 1 µg of Chl for each genotype. B) NPQ kinetics of selected KO mutants: WT is shown in black squares; *lhcsr1lhcsr2* KO in red circles; *psbs lhcsr1* KO in blue squares and *psbs lhcsr2* KO in green triangles. Actinic light was switch off after 8 min.

We then analyzed NPQ kinetics in all these plants (figure 1B). All double KO mutants showed smaller NPQ with respect to the starting single KO mutations (supplementary figure 2), further confirming that all proteins analyzed are active in NPQ. All mutants, however, show residual NPQ which is fast relaxed in the dark, which allows its identification as the qE component. These phenotypes allow to conclude that PSBS and both LHCSR isoforms are capable alone to activate an NPQ response. *psbs lhcsr2* KO plants, which express only LHCSR1, is the line retaining the strongest residual NPQ (Figure 1B), due to the fact that LHCSR1 is the most expressed isoform and is quantitatively more important for NPQ.

It is worth mentioning that all plants analyzed grown in control conditions did not show any major alteration in their photosynthetic apparatus compared to WT mosses, as evidenced by their Chl a/b, Chl/car values. Also PSII quantum yields, estimated by their Fv/Fm ratio of all the obtained lines are indistinguishable from the WT, as already evidenced in the case also of the triple mutant (Table 1 and (Alboresi *et al.*, 2010)).

	Fv/Fm	Chl a/b	Chl/Car
WT	0.82	2.43	3.99
psbs lhcsr1 KO	0.80	2.28	3.74
psbs lhcsr2 KO	0.82	2.46	3.84
lhcsr1 lhcsr2 KO	0.82	2.46	3.96

Table 1. PSII quantum yield and pigment content of *Physcomitrella patens* WT and NPQ double KO mutants grown in control conditions. PSII quantum yield (Fv/Fm) was evaluated in the different genotypes grown in control light conditions (40 µE). In the same plants Chl a/b and Chl/Car ratios were also evaluated. Values reported are mean of 3-5 independent experiments. SD is below 0.03 in the case of Fv/Fm, below 0.1 for Chl a/b and 0.3 for Chl/Car.

Generation of PSBS over-expressor lines: Physcomitrella is capable of a strong PSBS dependent NPQ in the absence of LHCSR

Results above showed that PSBS is capable alone of activating NPQ in *Physcomitrella* identifiable as the fast component qE. However, this is quantitatively small with respect to the LHCSR dependent component and to the vascular plants NPQ, raising the question if *Physcomitrella* PSBS protein is less active in NPQ with respect to vascular plants isoform and/or if *P. patens* photosynthetic apparatus is not able to fully undergo the structural rearrangements required for full NPQ activation (Kiss *et al.*, 2008; Betterle *et al.*, 2009). To answer this question we over-expressed PSBS in *lhcsr1lhcsr2* KO background (*lhcsr1 lhcsr2* KO-PSBS OE), using a construct which drives insertion of DNA in a region of *P. patens* genome (called BS213 (Schaefer & Zryd, 1997)) known to have no expressed proteins, thus avoiding inactivation of a endogenous gene. In this plasmid gene expression is under the control of a constitutive promoter derived from 35S (called 7113, (Mitsuhara *et al.*, 1996)). We obtained several stable resistant lines from the transformation, which were afterward analyzed for their level of PSBS accumulation and NPQ kinetics (figure 2).

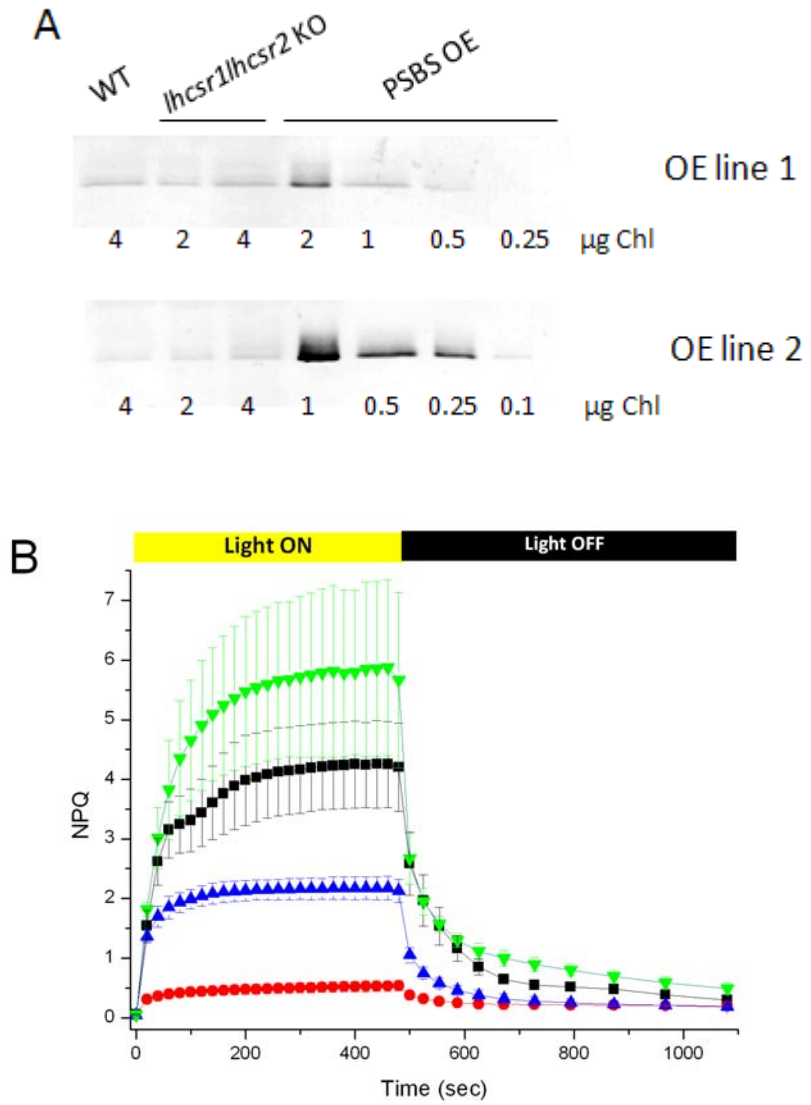


Figure 2. Western blot and NPQ phenotypes analyses of *lhcsr1 lhcsr2* KO-PSBS OE selected lines. A) Western blotting using antibody against PSBS. Different dilutions of each samples were loaded to better verify the amount of the over-expression of the protein, as antibody signal reaches a saturation when the quantity of the recognized protein is too high. We loaded 4 µg of Chls for WT, 2 and 4 µg of Chls for *lhcsr1lhcsr2*KO, for the *lhcsr1lhcsr2*KO-PSBS OE lines the µg of Chls loaded in each lane are reported in the figure. B) NPQ kinetics of selected mutants. Curves for WT, *lhcsr1lhcsr2*KO, *lhcsr1lhcsr2*KO-PSBS OE line 1 and 2 are shown in black, red, blue and green, respectively. Actinic light was switch off after 8 minutes. Data are presented as mean ± SD (n=3)

In several cases we found lines showing increased PSBS expression compared to the *lhcsr1lhcsr2* KO and WT, which instead present a similar level of PSBS accumulation. In figure 2A are reported western blotting analysis of a few independent lines, showing that these clones increased their PSBS accumulation in the thylakoids. We reported data from two lines, one with a small PSBS over-expression (line 1) and a second one presenting a huge protein accumulation (line 2), but additional lines showed similar phenotypes (not shown). The different expression of PSBS we obtained is likely due to

the fact that multiple insertion of transgene may occurred during homologous recombination in *Physcomitrella* (Kamisugi *et al.*, 2006), although ongoing experiments will verify this hypothesis.

All these plants in control conditions have unaffected Chl a/b and Chl/car values compared to WT and *lhcsr1 lhcsr2* KO mosses (supplementary figure 3) suggesting they do not show any major alteration in their photosynthetic apparatus. Also PSII quantum yields, estimated by Fv/Fm ratio, were very similar in all samples (supplementary figure 3).

Strong differences were instead found when their ability to activate NPQ was tested, finding in both *lhcsr1 lhcsr2* KO -PSBS OE lines an increase in NPQ capacity compared with the background. Interestingly, NPQ intensity depends on PSBS accumulation and in line 2, where PSBS is more abundant, NPQ is stronger, even more than in WT mosses. In all lines the NPQ is still identifiable as qE for its fast relaxation in the dark and for its dependence from nigericin (not shown). These results clearly demonstrate that the low PSBS-dependent NPQ capacity of *lhcsr1lhcsr2* KO is due to a lower expression of PSBS in *Physcomitrella* but the protein is fully capable of activating a strong NPQ even in the absence of LHCSR.

Generation of LHCSR over-expressor lines: LHCSR level determines NPQ level.

We showed above that the NPQ levels are determined by the PSBS accumulation. To verify if this was also the case for LHCSR, and if its accumulation modulated NPQ intensity in the absence PSBS, we over-expressed LHCSR in a *psbs* KO line.

As for the previous transformation, we obtained several independent lines showing an increased LHCSR expression compared with the *psbs* KO background (*psbs*KO-LHCSR OE) (figure 3A). As previously, these lines as well did not show major differences in pigment content and PSII quantum yield compared to the controls (supplementary figure 3). In this case, however, differently from PSBS OE mosses we did not observe any line with a major protein over-expression. This might be due to several reasons, for instance there might be a more efficient post-translational regulation for LHCSR than for PSBS, or more simply LHCSR level being already elevated in background cannot be much increased. Further analyses are needed to clarify this point.

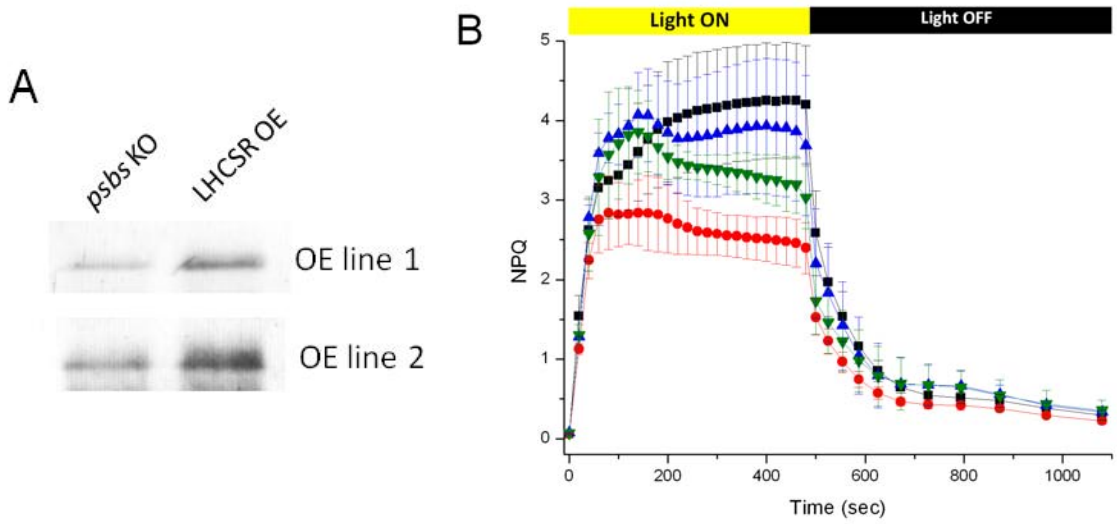


Figure 3. Western blot and NPQ phenotypes analyses of *psbs*KO-LHCSR OE selected lines. A) Western blotting using antibody against LHCSR in *psbs* KO background and selected OE lines. 1 µg of Chl was loaded in each case. B) NPQ kinetics of selected mutants. Curves for WT, *psbs* KO; *psbs*KO-LHCSR OE line 1 and 2 are shown in black, red, blue and green, respectively. Data are presented as mean ± SD (n=3)

When tested for their NPQ, also in LHCSR OE clones we found an increased NPQ capacity with respect to the genetic background (figure 3B). LHCSR OE mosses also show a peculiar NPQ kinetic, with a very rapid induction of NPQ within the first 2 minutes of light treatment, which then remained stable or slightly decreased: this is more similar to *psbs* KO, where only LHCSR is present, than to WT, suggesting it is somehow related to LHCSR-dependent NPQ. The fast relaxation in the dark allows identifying this thermal dissipation as qE component of NPQ, as in all the mutants we obtained before. NPQ values are higher in plants showing the larger LHCSR accumulation, demonstrating that also LHCSR level alone is regulating NPQ in *Physcomitrella* as we found above in the case of PSBS.

Complementation of psbs KO lines

Phenotypes of the previously presented KO and OE mosses allowed clarifying that both PSBS and LHCSR are able to induce qE alone in *Physcomitrella* and in both cases the level of accumulation of the protein in the thylakoids correlates with the NPQ capacity of each clone. These data support the idea that both proteins are capable alone of activating NPQ. However, they do not demonstrate if during NPQ in WT plants they interact or not. To verify this point we decided to complement *psbs* KO mosses with PSBS protein: if PSBS and LHCSR act independently, we expect to observe an increase in NPQ correlating with PSBS expression as in *lhcsr1lhcsr2* KO, while if they interact and enhance the

respective activity we expect a further increase. We choose in this case PSBS since it shows a larger range of expression levels, allowing a better verification of this point.

We obtained even in this case several stably resistant lines which also restored the expression of PSBS (*psbs* KO-PSBS OE lines). The pigment content showed as Chl a/b and Chl/Car ratios and the PSII quantum yield was similar to controls even in these clones. As well as in the previous case of PSBS OE in *lhcsr1lhcsr2* KO, we obtained lines with different expression level of PSBS and we present data from some selected lines, which are confirmed by other we obtained. Immunoblot analysis on thylakoids extracts of these lines showed that line 1 restored a level of expression of PSBS similar to WT mosses, while the other clones presented (lines 2 and 3) over-express the protein compared to WT (figure 4A).

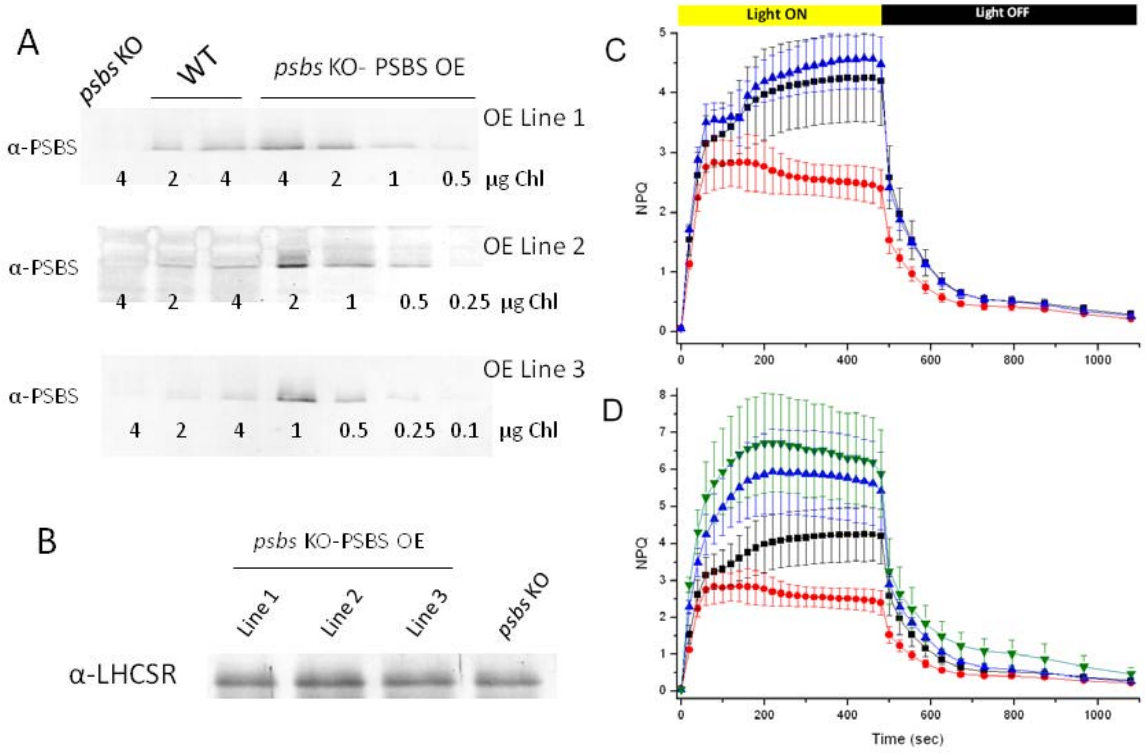


Figure 4. Western blot and NPQ phenotypes analyses of *psbs*KO-PSBS OE selected lines. A) Western blotting using antibody against PSBS. Different dilutions of each samples were loaded as in the case shown in figure 2. µg of Chls loaded in each case are reported above each lane in the figure. B) western blot against LHCSR in selected lines. 1µg of Chl was loaded for each sample. C-D) NPQ kinetics of selected mutants. C) Curves for WT, *psbs* KO and *psbs*KO-PSBS OE line 1 are shown in black, red and blue respectively. D) Curves for WT, *psbs* KO and *psbs*KO-PSBS OE line 2 and 3 are shown in black, red and blue and green, respectively. Note the different Y axis in C and D. Data are presented as mean ± SD (n=3)

When tested for their NPQ capacity (figures 4 C and D), the line 1, which presents a level of PSBS accumulation in the thylakoids similar to WT, complemented *psbs* KO phenotype showing an NPQ induction very close to WT mosses. Lines 2 and 3, instead, for which

western blot showed an higher accumulation of PSBS, are able to induce an NPQ response stronger than the WT, as likely expected from our previous results. LHCSR protein show no major differences in the expression of the protein in all genotypes tested (Figure 4B), demonstrating that this increased NPQ capacity was due only to the larger accumulation of PSBS and not to altered LHCSR expression.

DISCUSSION

PSBS and LHCSR are active simultaneously and independently in Physcomitrella

Analyses of the phenotypes of the different KO and OE mutants presented above highlight that each of the three proteins present in *Physcomitrella* (PSBS and two LHCSR isoforms) are able also to induce NPQ independently from the others. Although the effect on NPQ in control growth condition is stronger for LHCSR1 compared to the other two polypeptides, this is attributable to a different protein accumulation rather than higher protein activity. Consistently, in the several PSBS/LHCSR OE lines we obtained, we observed variable levels of NPQ amplitude correlating with PSBS/LHCSR accumulation, again suggesting that the low qE of *lhcsr1lhcsr2* KO and *psbs lhcsr1* KO is only due to a lower accumulation level of PSBS and LHCSR2 compared to LHCSR1.

PSBS/LHCSR OE lines (*lhcsr1lhcsr2* KO-PSBS OE and *psbs* KO-LHCSR OE) are able of a strong PSBS/LHCSR dependent NPQ in the complete absence of the other protein, further suggesting these two mechanisms of inducing NPQ in *Physcomitrella* are fully autonomous.

When LHCSR and PSBS are present together, total NPQ is roughly the sum of the individual contributions. Although NPQ values of different mutants cannot be summed, this nevertheless suggest that even if present at the same time PSBS and LHCSR works mostly independently the one from the other. Present data suggest the hypothesis that in WT mosses PSBS and LHCSR dependent NPQ are activated simultaneously but independently the one from the other, without strong interactions between these two mechanisms.

As a further confirmation, if the proteins interact somehow, we would expect that the modulation of one protein will affect to some extent the accumulation of the other one. Instead this was never observed in any KO or OE plants. This is particularly evident in PSBS complemented mosses (*psbs* KO-PSBS OE), where the strong increase in PSBS accumulation does not modulate at all LHCSR content. Overall, our findings demonstrated that they not only are able to work autonomously but they do not

influence each other *in vivo*, strongly suggesting PSBS and LHCSR are active in NPQ simultaneously but independently.

Such a conclusion also support the hypothesis that PSBS and LHCSR mechanisms in activating NPQ are different with the former suggested to act as an inducer of a quenched state in antenna system during NPQ (Bonente *et al.*, 2008; Betterle *et al.*, 2009; Kereiche *et al.*, 2010), while LHCSR is suggested to dissipate energy directly (Peers *et al.*, 2009; Bonente *et al.*, 2011). We also observed small differences in NPQ kinetic when only PSBS or LHCSR are present, which are also consistent with a different mechanism with slightly different activation rates.

Regulation of PSBS and LHCSR accumulation modulates NPQ amplitude.

In this work we obtained several mutant lines for PSBS and LHCSR proteins, with different expression level of these polypeptides. In all plants analyzed here we clearly found a correlation between NPQ and PSBS or LHCSR accumulation. This was true either in the case of lines where only PSBS/LHCSR were present, as well as in PSBS complemented mosses, where NPQ amplitude is modulated by the expression level of PSBS, without affecting the one of endogenous LHCSR. This suggests that in *Physcomitrella* NPQ capacity is determined by the accumulation of these polypeptides, and thus these data confirm our previous results on acclimated plants (see chapter 3). The regulation of LHCSR and PSBS accumulation is a method for *Physcomitrella* plants to modulate the NPQ capacity according to environmental conditions.

This finding is not only restricted to *P. patens*, but is consistent with previous data for plants and green algae. In fact, analogous results were obtained for *Arabidopsis* where increased PSBS yielded higher NPQ (Li *et al.*, 2002a; Ballottari *et al.*, 2007), and also in *Chlamydomonas* increased LHCSR expression yielded increased NPQ. In this alga LHCSR is only accumulated in HL conditions, suggesting little need for NPQ in CL conditions: when instead this is needed, LHCSR accumulation assures this possibility (Peers *et al.*, 2009).

Evolution of PSBS and LHCSR dependent NPQ mechanisms

All data present in the literature thus suggest a picture for evolution of NPQ mechanism in photosynthetic organisms going from a LHCSR to a PSBS dependent mechanism moving in the phylogenetic tree from algae to land plants (Li *et al.*, 2000; Peers *et al.*, 2009). Results of this work also suggest that in the transition from one mechanism to the other there was an overlapping of these two mechanisms. Although they are present simultaneously, they are likely autonomous, thus allowing for the evolution of PSBS

dependent NPQ while LHCSR still ensures enough photoprotection. We detailed discuss the evolution of NPQ process from algae to plants in chapter 7.

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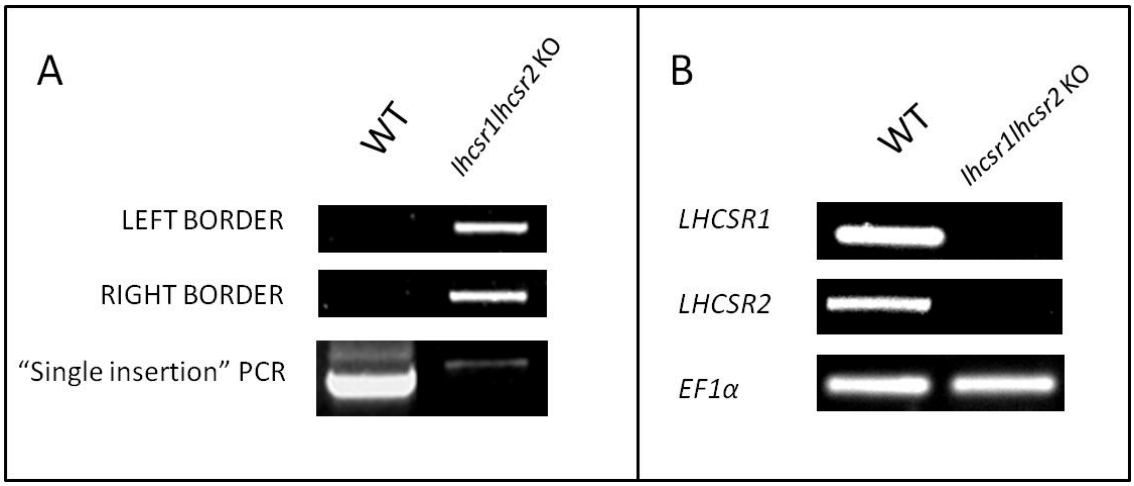
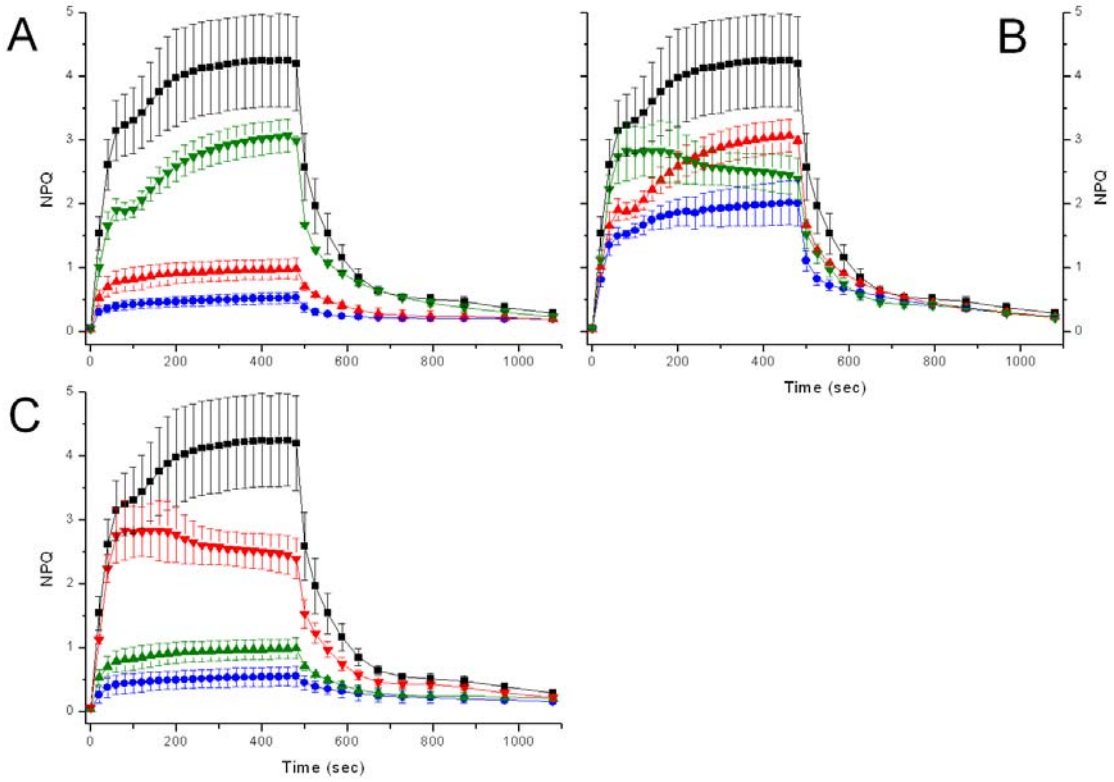
SUPPLEMENTARY INFORMATIONS

Supplementary figure 1. KO mutant characterization: *lhcsr1lhcsr2* KO is reported as example. A) verification of DNA insertion in the genome by amplification of the left and right borders. One primer anneals in the genomic locus while the other within the targeting construct. Amplification is possible only in transgenic lines where homologous recombination occurred. Mutants carrying a single integration are then identified by the different size of the amplified band with respect to WT ("single insertion PCR"). B) Evaluation of LHCSR gene expression assessed by RT-PCR in WT and mutant line. Elongation Factor-1 alpha (EF1α) is also reported as a control. The primer used for the PCR assays were the same as in chapter 2.



Supplementary figure 2: **NPQ kinetics of *psbs* and/or *lhcsr* KO lines.** Each double KO (blue circles) is presented with the respective single KO combined (in red triangles the one used as a background of the transformation). A) *lhcsr1lhcsr2* KO in blue; *lhcsr1* KO in red; *lhcsr2* KO in green; B) *psbs lhcsr2* KO in blue, *lhcsr2* KO in red and *psbs* KO in green; C) *psbs lhcsr1* KO in blue, *psbs* KO in red and *lhcsr1* KO in green. In each graph also WT is reported in black squares. Clearly each double KO reduced its NPQ capacity compared with both the corresponding single KO. It is worth mentioning that in the case of *lhcsr1lhcsr2* KO we obtained clones also transforming *lhcsr2* KO as a background with KO construct for LHCSR1, opposite to the clone showed in this work, which present the same NPQ phenotypes as the one presented here, further confirming that the observed phenotype is due to the depletion of the proteins and not to secondary effects, as we observed in the case of *psbs* KO generated with vectors carrying different resistance cassettes presented in (Alboresi *et al.*, 2010). Data are presented as mean ± SD (n=3). Actinic light was switch off after 480 sec.



Supplementary Figure 3. PSII quantum yield and pigment content of *Physcomitrella patens* WT and over-expressor mutants grown in control conditions. PSII quantum yield (Fv/Fm) was evaluated in the different genotypes grown in control light conditions (40 µE). In the same plants Chl a/b and Car/Chl ratios were also evaluated. Values reported are mean of 3-5 independent experiments. SD is below 0.03 in the case of Fv/Fm, below 0.1 for Chl a/b and 0.3 for Chl/Car.

	Fv/Fm	Chl a/b	Chl/Car
WT	0.82	2.43	3.99
psbs KO (P81)	0.83	2.40	4.02
*psbs*KO-PSBS OE line 1	0.81	2.47	4.21
*psbs*KO-PSBS OE line 2	0.81	2.45	4.15
*psbs*KO-PSBS OE line 3	0.79	2.49	3.85
*psbs*KO-LHCSR OE line 1	0.81	2.49	4.18
*psbs*KO-LHCSR OE line 2	0.81	2.52	4.07
lhcsr1 lhcsr2 KO	0.083	2.46	3.96
*lhcsr1lhcsr2*KO-PSBS OE line 1	0.82	2.45	4.13
*lhcsr1lhcsr2*KO-PSBS OE line 2	0.81	2.50	4.17

CHAPTER 5

PSBS N AND C TERMINAL ARE NOT ESSENTIAL FOR NPQ ACTIVATION IN *PHYSCOMITRELLA PATENS*

PSBS N AND C TERMINAL ARE NOT ESSENTIAL FOR NPQ ACTIVATION IN *PHYSCOMITRELLA PATENS*

ABSTRACT

PSBS plays a major role in NPQ in vascular plants as in the moss *Physcomitrella patens*. PSBS in all plants showed conserved basic residues and acidic residues respectively at the N and C terminal. In order to verify their possible role in NPQ activation we here complemented *psbs* KO mutant of the moss *Physcomitrella patens* using *Pp*PSBS WT and two truncated forms at respectively N and C terminal. All complemented plants showed restored NPQ, whose extent depended mainly on the protein expression level and not on the eventual presence of a deletion. These results suggest that, although conserved, these charged residues in PSBS does not play a major role in NPQ. N-terminal residues might be involved in facilitating the protein insertion into the thylakoid membrane.

INTRODUCTION

In previous chapters we extensively discussed how in plants NPQ relies on the presence of a protein called PSBS (Li *et al.*, 2000). PSBS is an Lhc-like polypeptide and thus it shares some similarity with proteins composing the photosystems antenna system (Lhc). PSBS, however, has four trans-membranes helices instead of the three normally found in antenna proteins and it is believed to be unable to bind pigments (Dominici *et al.*, 2002; Crouchman *et al.*, 2006; Bonente *et al.*, 2008). PSBS is activated by the protonation of two glutamate residues, E122 and E226, when lumenal pH becomes acidic (Li *et al.*, 2000; Li *et al.*, 2002; Li *et al.*, 2004). PSBS, once activated, induces a decrease in excited states lifetime in the pigment-binding subunits of the antenna system consequent to a reorganization of photosystem subunits in the thylakoid membranes (Betterle *et al.*, 2009; Bonente *et al.*, 2008; Horton *et al.*, 2008; Kiss *et al.*, 2008)

We recently demonstrated that PSBS is active in triggering NPQ also in the moss *Physcomitrella patens*, and that this organism is the only known plants where both LHCSR, the polypeptides involved in NPQ in many algae(Peers *et al.*, 2009) (Zhu & Green, 2010), and PSBS proteins are active in NPQ (Chapters 2-4) (Alboresi *et al.*, 2010). We here exploited *P. patens* genetic manipulability to access the structural requirement for PSBS activity. In fact, while it is clear that PSBS activation is driven by protonation of lumenal glutamates, upon activation PSBS must interact with other proteins of the photosynthetic apparatus, possibly also after dimerization (Bergantino *et al.*, 2003). It is still not clear which parts of the protein are responsible for this activity. By analyzing the

protein sequence we identified that the N and C terminal part of PSBS have conserved basic and acidic residues. To verify if these residues might be involved in protein interaction or dimerization we complemented *psbs* KO mutant with PSBS either WT or in N/C terminal truncated form. Results showed that in all three cases we observed restoration of NPQ capacity which was correlated with protein accumulation and not with the presence/absence of the deletion, suggesting that these parts of the protein are not fundamental for PSBS activity.

MATERIALS AND METHODS

Sequence retrieval and analysis. PSBS sequence from *Physcomitrella patens* were retrieved from genome database (http://genome.jgi-psf.org//Phypa1_1/Phypa1_1.home.html), as described in (Alboresi *et al.*, 2008). Sequences from other plants were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence alignments were generated using clustalW and manually corrected using BioEdit. Sequence logos were built using Weblogo server (http://weblogo.berkeley.edu/logo.cgi).

Plant material. Protonemal tissue of *P. patens*, Gransden wild-type strain, *psbs* KO (Alboresi *et al.*, 2010) and PSBS OE lines were grown on minimum $PpNO_3$ media (Asthon *et al.*, 1979) 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, PRESTON, UK) as previously described (Alboresi *et al.*, 2008). 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^{-2} s^{-1}.

Protoplast transformation and OE generation. For the generation of OE mosses we used as starting material cDNA obtained from *P. patens* protonema grown in control conditions. Complete or N-terminal or C-terminal deleted coding sequences for PSBS were amplified by PCR (for the primers employed for the amplification see table 1 of the introduction) and successively cloned into pMAK1 vector (kindly provided by Prof. Takashi Murata, NIBB, Okazaki, Japan) using XhoI/HpaI restriction enzymes. *P. patens* transformation was performed as in (Schaefer & Zryd, 1997) with minor modifications, as reported in Chapter 2.

NPQ measurements. In vivo chlorophyll fluorescence of *P. patens* grown 10 days in minimum medium was measured at room temperature with Dual PAM-100 fluorometer (Walz), using a saturating light at 6000 µE m^{-2} s^{-1} and actinic light of 830 µE m^{-2} s^{-1}. Before measurements, plates were dark-adapted for 40 minutes at room temperature.

The parameters Fv/Fm and NPQ were calculated as (Fm-Fo)/Fm, and (Fm-Fm')/Fm' (Genty *et al.*, 1989).

Thylakoids extraction, SDS-PAGE and Western Blotting analyses. Thylakoids from protonemal tissue (10 days old plants grown in minimum $PpNO_3$) were prepared using an *Arabidopsis* protocol with minor modifications (Alboresi *et al.*, 2008). For immunoblotting analysis, following SDS-PAGE, proteins were transferred onto a nitro-cellulose membrane (Pall Corporation) using a blot system from Amersham and detected with specific home-made polyclonal antibodies.

RESULTS & DISCUSSION

Alignment of PSBS protein from different plants and algae shows conserved features at N and C terminal.

Genes encoding for PSBS polypeptides are present in all plants where extensive sequences data are available. We here retrieved sequences from several species and we analyzed PSBS aminoacid sequences in order to identify key conserved features, which might be involved in protein activity. In figure 1 we report the alignment of PSBS mature sequences from different plants, either vascular (*Arabidopsis thaliana, Zea mays, Oryza sativa, Populus trichocarpa, Vitis vinifera, Nicotiana benthamiana*) or non vascular (*Physcomitrella patens, Selaginella moellendorphii*). Alignment is represented as a Seqlogo which is helpful in evidencing conserved residues (Figure 1). As expected, we found that transmembrane helices are particularly conserved while hydrophilic parts show more variability. Among the latter we observed that N-terminus showed a remarkable conservation of positively charged residues. Also in the C terminal part we observed several residues with a conserved charge, although in this case it is a negative one (Figure 1).

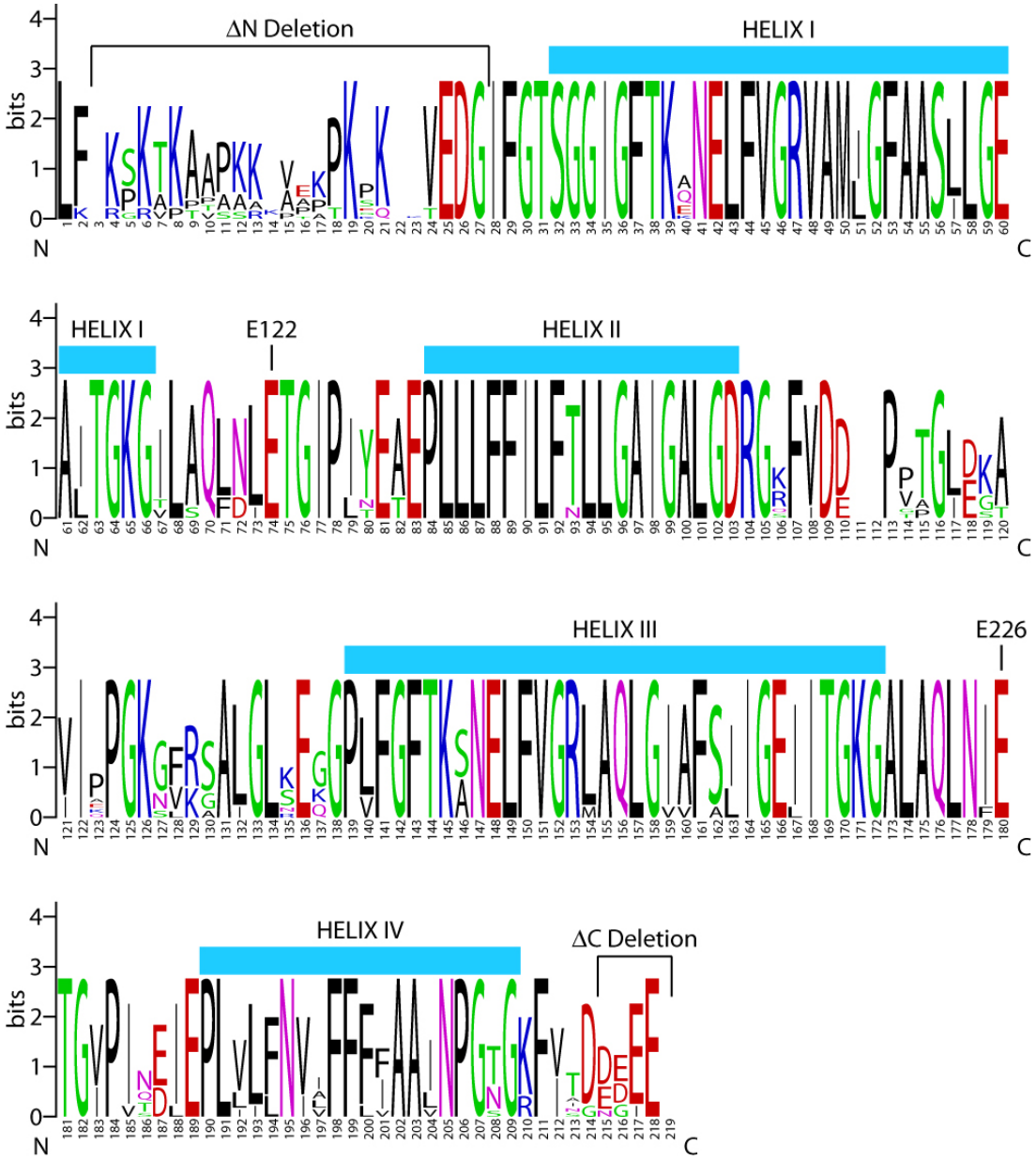


Figure 1. PSBS sequence conservation in different plants species. PSBS mature sequences from different plants were aligned and protein conservation shown as SeqLogo. Position of transmembrane helices, Glutamate residues fundamental for activity (E122 and E226) and residues deleted in ΔN and ΔC-PSBS are shown

Complementation of psbs KO phenotype with WT and truncated PpPSBS protein

As mentioned above, PSBS function is driven by interaction with other proteins and, possibly, also by dimerization which might involve these charged residues identified at N and C terminal of the protein. To test this hypothesis we exploit the availability of a knock-out mutant for PSBS (*psbs* KO) of the moss *Physcomitrella patens* and the genetic manipulability of this organism to generate plants complemented with the PSBS protein either in the WT or N/C terminal truncated from (respectively WT, ΔN and ΔC-PSBS from

now on). The exact point of truncation is reported in figure 1. It is worth mentioning that in the case of ΔN-PSBS we retained the region corresponding to the transit peptide to ensure that the protein is correctly inserted into thylakoids where it can be active and we deleted the N- terminal aminoacids of the mature protein (aminoacids 3-27 of the mature protein, as reported in figure 1; for a detailed description of the PCR procedure to obtain ΔN-PSBS coding sequence, see table 1 of the introduction).

For the transformation we cloned the PSBS cDNA in a vector, called pMAK1, which drives the insertion of DNA in a region of *Physcomitrella* genome (called BS213 (Schaefer & Zryd, 1997)) known to encode for any expressed proteins, in order to avoid the inactivation of a endogenous gene. In this vector gene expression is under the control of a strong constitutive promoter (7113, modified from 35S (Mitsuhara *et al.*, 1996)). After the transformation and two selection rounds we obtained several independent resistant colonies, which were further characterized for their phenotype.

First of all we tested all lines for the PSBS protein accumulation: as shown in figure 2 in the case of WT-PSBS we obtained several lines with restored expression of the protein. Expression levels are variable in different lines, with some plants showing protein accumulation comparable to WT (lines OE 5 and 79) and others where PSBS levels in the thylakoid membranes are far higher (lines OE 8 and 40). The strong expression in these lines is likely due to insertion of multiple copies of the transgene in the genome which drives to a more intense translation (Kamisugi *et al.*, 2006) .

PSBS expression have been identified also for lines complemented with ΔN-PSBS (figure 2). In this case ΔN-PSBS protein has a smaller molecular weight with respect to WT protein, as expected since it weights 20.1 KDa vs. 22.3 KDa of the WT form. The molecular weight observed, together with the fact that the protein is found in thylakoids, suggests that protein is successfully imported in the chloroplast and the transit peptide cleaved. In the case of this mutant we did not observe any clone where the protein was massively accumulated as in WT form.

In the case of lines complemented with ΔC-PSBS as well we observed protein accumulation using specific antibodies, but we observed only a small weight difference, as expected considering its 21.7 KDa mass. In this case, as for WT, in a few cases we observed lines with very abundant expression levels (ΔC-PSBS line OE 79; figure 2).

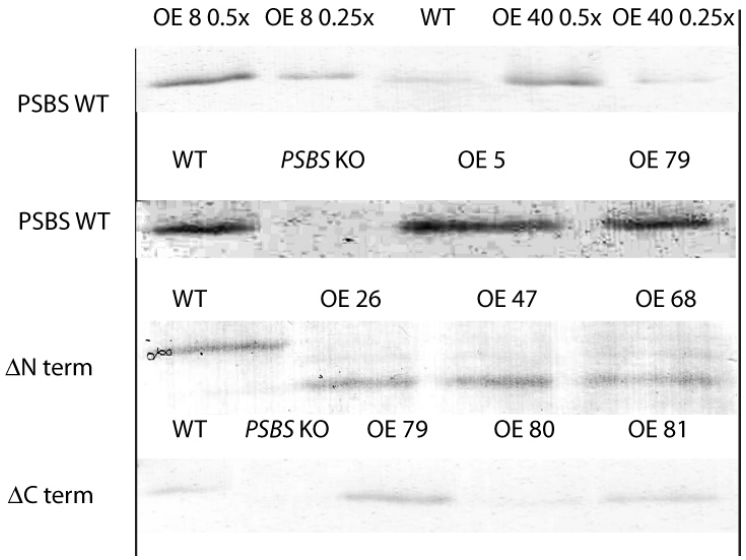


Figure 2. PSBS accumulation in complemented lines. Western blotting using antibody against PSBS. Several complemented lines with WT, ΔN and ΔC PSBS are shown. In the case of PSBS WT 4 µg of Chl were loaded for each sample, with the exception of the over-expressing lines 8 and 40 for which 2 and 1 µg of Chl were loaded in two different lanes. In the case of ΔN PSBS, we loaded around 8 µg of Chl in each lanes, while for ΔC PSBS we loaded 4 µg of Chl for each sample. In each blot also thylakoid extracts (4 µg of Chl) from WT *Physcomitrella* (WT) or *psbs* KO line were loaded as controls.

PSBS N and C-terminal do not play a major role in NPQ activation.

Previous results showed that we obtained several lines with different accumulation levels of PSBS in the WT and truncated forms. We tested these lines for their NPQ capacity to access if the accumulated protein was indeed functional (figure 3). As shown by the kinetics reported in figure 3A in the case of PSBS WT we observed a clearly restored NPQ in complemented plants with respect to the *psbs* KO mutant, where residual NPQ only relies on LHCSR. In plants where PSBS accumulation is similar to WT we observed also comparable NPQ levels. Interestingly, however, where PSBS is massively accumulated (OE lines 8 and 40) we also observed very strong NPQ (figure 3A; see also chapter 4).

In the case of plants complemented with ΔN-PSBS, lines showing significant protein accumulation also have stronger NPQ with respect to the background *psbs* KO (figure 3B). These results thus suggest that ΔN-PSBS is indeed active in NPQ, although it seems to be accumulated in the thylakoids to lower amounts with respect to the WT protein. This might be due to the fact that any selected lines carried multiple transgene insertion and the consequent abundant transcription. However, since we did not observe any difference in transformation efficiency between all constructs presented here, we support an alternative hypothesis which the N terminal truncated form might be less efficiently inserted in the thylakoids membrane with respect to WT. In fact, it has

already been shown that positively charged residues in the N-term are important to drive the insertion of Lhc proteins in the thylakoids (Kim *et al.*, 1999).

In the case of ΔC-PSBS plants, we also observed restored NPQ. Also in these plants, as in WT-PSBS, complemented plants with higher protein content also have stronger NPQ showing a correlation between PSBS accumulation and NPQ capacity (figure 3C). These results clearly show that the truncated form is active in NPQ as well as the WT.

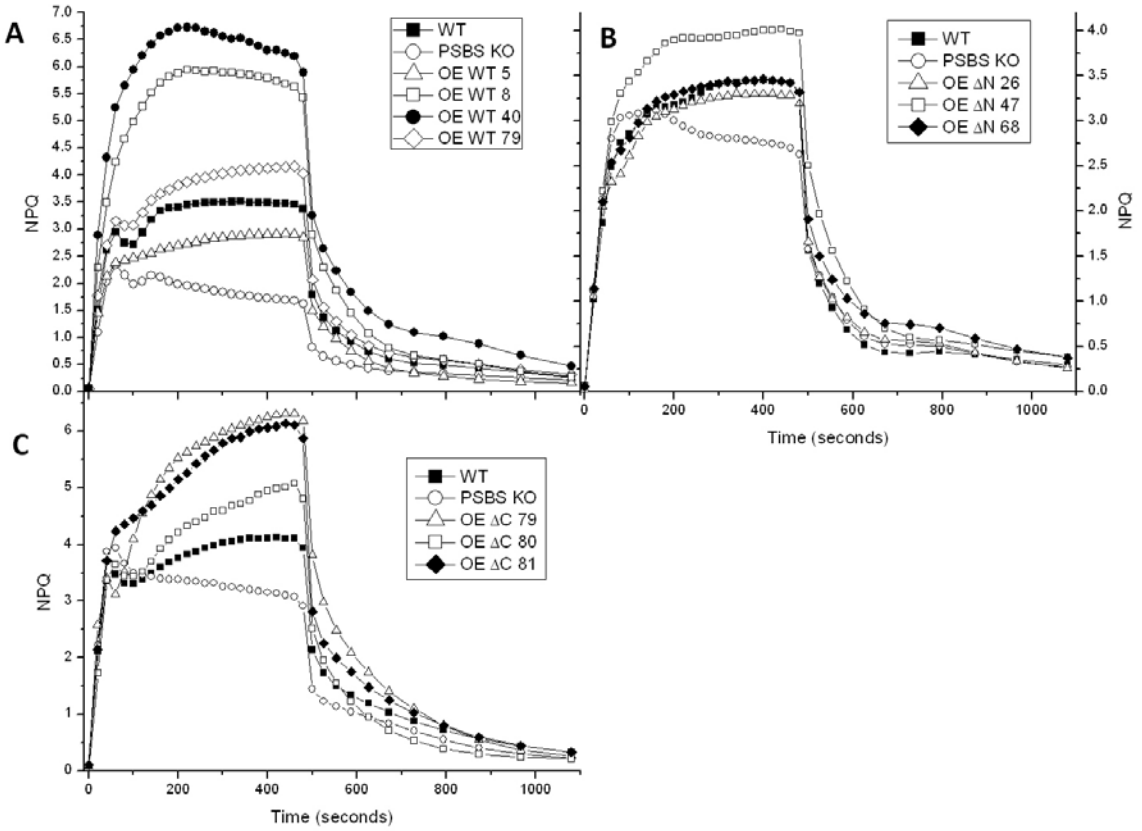


Figure 3. NPQ kinetics in complemented lines. NPQ kinetics measured for WT mosses, *psbs* KO and all the complemented plants are shown. WT PSBS protein is shown in panel A, while lines complemented with ΔN and ΔC PSBS are respectively shown in B and C. The kinetics of WT and *psbs* KO plants are a little different in the different panels due to slightly different growth conditions used in each case.

Conclusions

In conclusion presented results showed the *Physcomitrella patens* is a valuable model for the study of PSBS structure-function relationship *in vivo*, thanks to the possibility of complementing mutant plants with mutated forms of the proteins. We here tested the N and C terminal truncated form of PSBS protein and results showed that these regions are not fundamental for its activity. Most likely, thus, protein interactions believed to be fundamental for PSBS activity are mediated by other parts of the protein, as the transmembrane regions.

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CHAPTER 6

ROLE OF ZEAXANTHIN IN *PHYSCOMITRELLA PATENS* NPQ

ROLE OF ZEAXANTHIN IN *PHYSCOMITRELLA PATENS* NPQ

ABSTRACT

Plants live in a variable environment where light intensity can change very rapidly, leading to photodamage in plant cells. Several protection mechanisms are involved in the protection of photosynthetic apparatus from light induced damage. Zeaxanthin synthesis is induced in high light and this carotenoid plays a central role in photoprotection. Among its different effects, at least in *Arabidopsis* it is known to enhance NPQ. We here analyzed the influence of zeaxanthin in NPQ of the moss *Physcomitrella patens*. Results presented showed that although NPQ is induced also in the absence of zeaxanthin, the synthesis of this carotenoid is fundamental for the activation of a strong NPQ in this moss. Zeaxanthin influences similarly PSBS and LHCSR activities but in their absence no fast NPQ is activated.

INTRODUCTION

As already discussed in detail in previous chapters, plants live in a variable environment and evolved multiple mechanisms to protect their photosynthetic apparatus from oxidative damage (see chapter 1, section 3). In addition to PSBS and LHCSR proteins activities analyzed in previous chapters, also carotenoids, and zeaxanthin in particular, are involved in the protection of the photosynthetic apparatus from reactive oxygen species (ROS) formation.

Zeaxanthin is synthesized in high light conditions from violaxanthin by the enzyme Violaxanthin de-epoxidase (VDE) (Arnoux *et al.*, 2009), and it plays a central role in photoprotection in chloroplast, being involved in several protection mechanisms (Havaux & Niyogi, 1999; Jahns *et al.*, 2009). In fact, in the model vascular plant *Arabidopsis thaliana* zeaxanthin contributes to the heat dissipation of excess excitation energy in the qE mechanism by enhancing PSBS-dependent NPQ induction (Niyogi *et al.*, 1998; Holt *et al.*, 2005; Jahns *et al.*, 2009). It is also likely involved in slower photoprotection processes components of NPQ (Dall'Osto *et al.*, 2005; Nilkens *et al.*, 2010). Moreover, zeaxanthin bound to antenna complexes is active in the scavenging of Chl triplets (Mozzo *et al.*, 2008) and, when accumulated free in the thylakoids membrane, it is an effective antioxidant for the scavenging of ROS in the membranes to avoid lipids peroxidation (Havaux & Niyogi, 1999; Demmig-Adams & Adams, III, 2002; Havaux *et al.*, 2007; Jahns *et al.*, 2009).

Xanthophyll cycle is well distributed in many other photosynthetic eukaryotes, like green algae but also diatoms (Coesel *et al.*, 2008). In particular, in diatoms the activation of the xanthophyll cycle has a major role in NPQ (Grouneva *et al.*, 2008). On the contrary, it appears that zeaxanthin has a smaller influence on NPQ in *Chlamydomonas* cells, since the phenotype of *Chlamydomonas npq1* (the mutant unable to produced zeaxanthin) grown in control condition is weaker compared to *Arabidopsis npq1* (Niyogi *et al.*, 1997a; Niyogi *et al.*, 1998). Zeaxanthin thus seems to have a different influence in NPQ in different organisms, which might be correlated with the fact that NPQ is PSBS or LHCSR dependent.

Physcomitrella patens is the ideal organism to access if indeed zeaxanthin has different effect on PSBS and LHCSR, since it has both proteins. We here analyzed the influence of zeaxanthin production in *P. patens* NPQ. Results showed that zeaxanthin synthesis strongly increases NPQ intensity, enhancing both PSBS and LHCSR effects. In the absence of these proteins, however, zeaxanthin alone is unable to induce any quenching.

MATERIALS AND METHODS

Plant material. Protonemal tissue of *P. patens*, Gransden wild-type strain, *psbs* and/or *lhcsr* KO lines (Alboresi *et al.*, 2010) and *vde* KO lines (*vde* KO lines were kindly provided by Alessandro Alboresi, University of Verona) were grown on minimum $PpNO_3$ media (Asthon *et al.*, 1979) 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, PRESTON, UK) as previously described (Alboresi *et al.*, 2008). 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^{-2} s^{-1} (CL). In the case of light treatments, 5 days old plants were transferred to 10 µE m^{-2} s^{-1} (low light, LL) or to 450 µE m^{-2} s^{-1} (high light, HL). *Arabidopsis* WT (ecotype Columbia) and *npq1* plants (Niyogi *et al.*, 1998) were grown at 100 µE m^{-2} s^{-1}, 8 h light/16 h dark photoperiod.

NPQ measurements. *In vivo* chlorophyll fluorescence of *P. patens* grown 10 days in minimum medium was measured at room temperature with Dual PAM-100 fluorometer (Walz), using saturating light at 6000 µE m^{-2} s^{-1} and actinic light of 830 µE m^{-2} s^{-1}. Before measurements, plates were dark-adapted for 40 minutes at room temperature. The parameters Fv/Fm and NPQ were calculated as (Fm-Fo)/Fm, and (Fm-Fm')/Fm' (Demmig-Adams *et al.*, 1996). Inhibitor treatment was performed as in chapter 3: *P. patens* colonies were incubated with dithiotreitol (DTT) 5 mM in 150 mM sorbitol, 10 mM

HEPES (pH 7.5) buffer during the 40 minutes dark-adaptation at RT before measurements. Control samples were incubated with the HEPES/sorbitol buffer only.
In the case of *Arabidopsis*, detached leaves were measured with 1200 µE m^{-2} s^{-1} of actinic light after 40 min of dark adaptation at RT. For DTT treatment, leaves were infiltrated with DTT 5mM in HEPES/sorbitol buffer, and incubated in the same solution during dark adaptation as well. Control samples were infiltrated with the buffer only, as for *Physcomitrella*.

RESULTS & DISCUSSION

Zeaxanthin effect in Physcomitrella NPQ

As already reported (see chapter 3), *Physcomitrella* exposed to strong light have altered carotenoid content with the activation of zeaxanthin synthesis due to VDE activation (Arnoux *et al.*, 2009).
In order to investigate the influence of the synthesis of zeaxanthin on NPQ, we treated WT plants with a VDE inhibitor, DTT, before NPQ measurements: DTT inhibits zeaxanthin synthesis from violaxanthin induced by actinic light and thus allows identifying which fraction of NPQ activation is due to the accumulation of this carotenoid.
As shown in figure 1A, DTT has a strong effect on NPQ kinetics in WT mosses, drastically reducing its amplitude and demonstrating that this carotenoid is fundamental for its full activation in *Physcomitrella*. It is worth mentioning, however, that residual NPQ is identifiable as the fast component qE, since it has fast activation and relaxation. In figure 1B is reported the same experiment in the model plant *A. thaliana*: NPQ is similarly reduced after DTT treatment with respect to control conditions, showing that zeaxanthin is necessary for a complete activation of NPQ also in this organism. In *Arabidopsis* plants the effect of DTT is very close to the phenotype of *npq1* mutant, which is affected in VDE activity and are unable to produce zeaxanthin from violaxanthin (Niyogi *et al.*, 1998), demonstrating that DTT treatment is a valuable tool to asses zeaxanthin involvement in NPQ. DTT secondary effects, if present, have no major influence in the few minutes of the NPQ measurement.

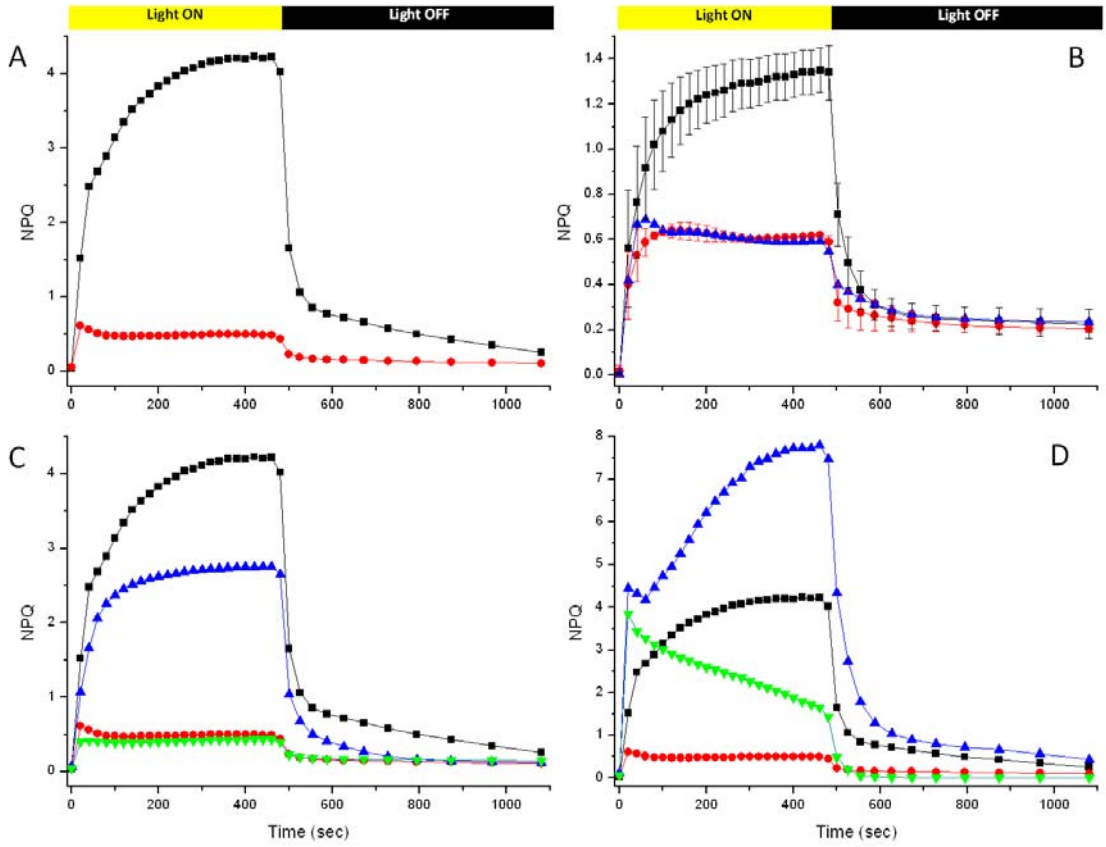

Figure 1. DTT treatment in *Arabidopsis* and WT mosses acclimated to different light conditions.
A) DTT effect in WT mosses (grown in control light conditions, CL). WT sample treated with the buffer (control) is shown in black squares, while DTT treated colonies in red circles. B) *Arabidopsis*: black squares, WT leaves treated with buffer only (control), red circles, DTT treated leaves (WT), blue triangles, *npq1* leaves. C) Effect of DTT on Low light (LL) grown mosses: black and red kinetics are the same of figure 1A, LL sample treated with buffer is shown in blue, LL DTT treated in green D) Effect of DTT in high light (HL) acclimated plants: black and red kinetics are the same of figure 1A, HL sample treated with buffer is shown in blue triangles, HL DTT treated in green triangles. In all cases actinic light is switch off after 8 min. Note the different y scale in the different panels.

In *Physcomitrella patens* we also evaluated the effect of DTT treatment on NPQ capacity of acclimated samples (figure 1C and D). In low light (LL) growth conditions zeaxanthin is absent or very low as in control light (CL) grown plants (figure 1C) and, consistently, DTT has a very similar effect on NPQ kinetics in LL and CL samples. On the contrary, high light (HL) acclimated plants have a pre-existing zeaxanthin accumulated during growth (5.5 Zeaxanthin molecules per 100 Chls, see Chapter 3). Here DTT treated plants show a strong and fast induction of NPQ which however is not sustained (figure 1D). These kinetics thus suggest that zeaxanthin accumulated in strong light conditions is not sufficient for a full NPQ induction, but additional zeaxanthin has to be synthesized after light is switched on to achieve a complete activation of the process.

An alternative method to analyze the effect of zeaxanthin accumulation is to perform two successive NPQ measurements separated by a short dark adaptation (10 minutes).

During this short dark interval electron chain transporters are re-oxidized and ΔpH dissipated, but this time interval is not sufficient for the complete conversion of zeaxanthin accumulated during the first light treatment back to violaxanthin since de-epoxidation reaction is relatively slow (Jahns *et al.*, 2009). Thus, in the second measurement zeaxanthin is already present and because of that in *Arabidopsis* it has been shown that NPQ activation in the second measurement is faster than in the first one (Andersson *et al.*, 2001). We observed also in *Physcomitrella* the same acceleration in all the condition tested with WT mosses, irrespective to the final NPQ value (figure 2A), confirming that zeaxanthin accumulation enhance NPQ in *P. patens* as well. When normalized to the maximal NPQ values of the first light period, the kinetics of LL, CL and HL samples are very similar, again suggesting that the fraction of zeaxanthin involved in qE has to be newly synthesized upon the exposure to strong light, while the already present zeaxanthin is likely involved in other protection processes.

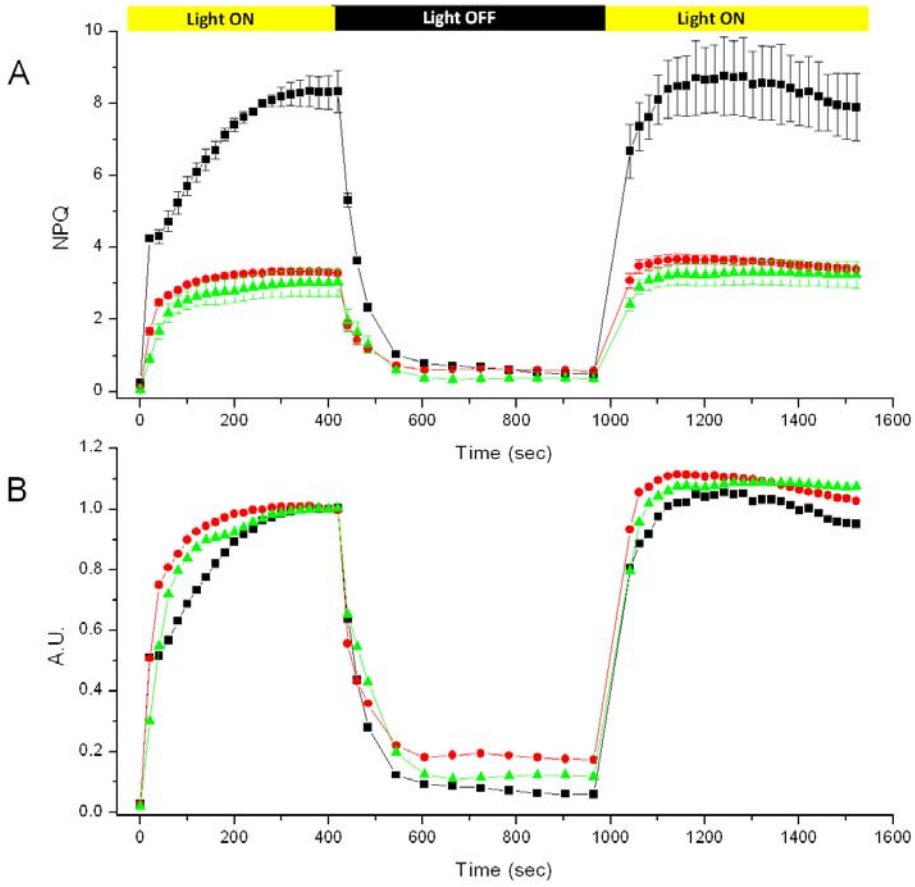

Figure 2: Double kinetics in WT mosses acclimated to different light conditions. Samples are exposed to actinic light for 8 minutes, followed by 10 minutes of dark recovery, as in the other kinetics presented. Here at the end of the dark period actinic light is switch on again. A) NPQ kinetics; B) NPQ kinetics normalized to the maximal value reached at the end of the first actinic light period. HL, CL and LL acclimated mosses are shown in black squares, red circles and green triangles, respectively.

Zeaxanthin effect in Physcomitrella PSBS and LHCSR mutants

Once established that zeaxanthin is needed for a complete induction of NPQ, we also wanted to analyze if its accumulation was influencing specifically PSBS and/or LHCSR dependent NPQ. As KO mutants phenotype clearly demonstrates (chapters 2-4), in fact, PSBS and LHCSR are both fundamental proteins for NPQ activation in *Physcomitrella,* but their activities are independent. Zeaxanthin thus might affect majorly one with respect to the other. The residual NPQ after DTT treatment in WT mosses, in fact, is very close to residual qE in *Arabidopsis* leaves treated with DTT, where NPQ is only PSBS dependent.

To verify if the effect of DTT in *Physcomitrella* can be due to different sensitivity of PSBS and LHCSR activities to zeaxanthin, DTT treatment was applied to *psbs* and/or *lhcsr* KO mutants described in Chapters 2 and 4. We observed a strong effect of DTT treatment in all genotypes tested but the triple KO (figure 3).

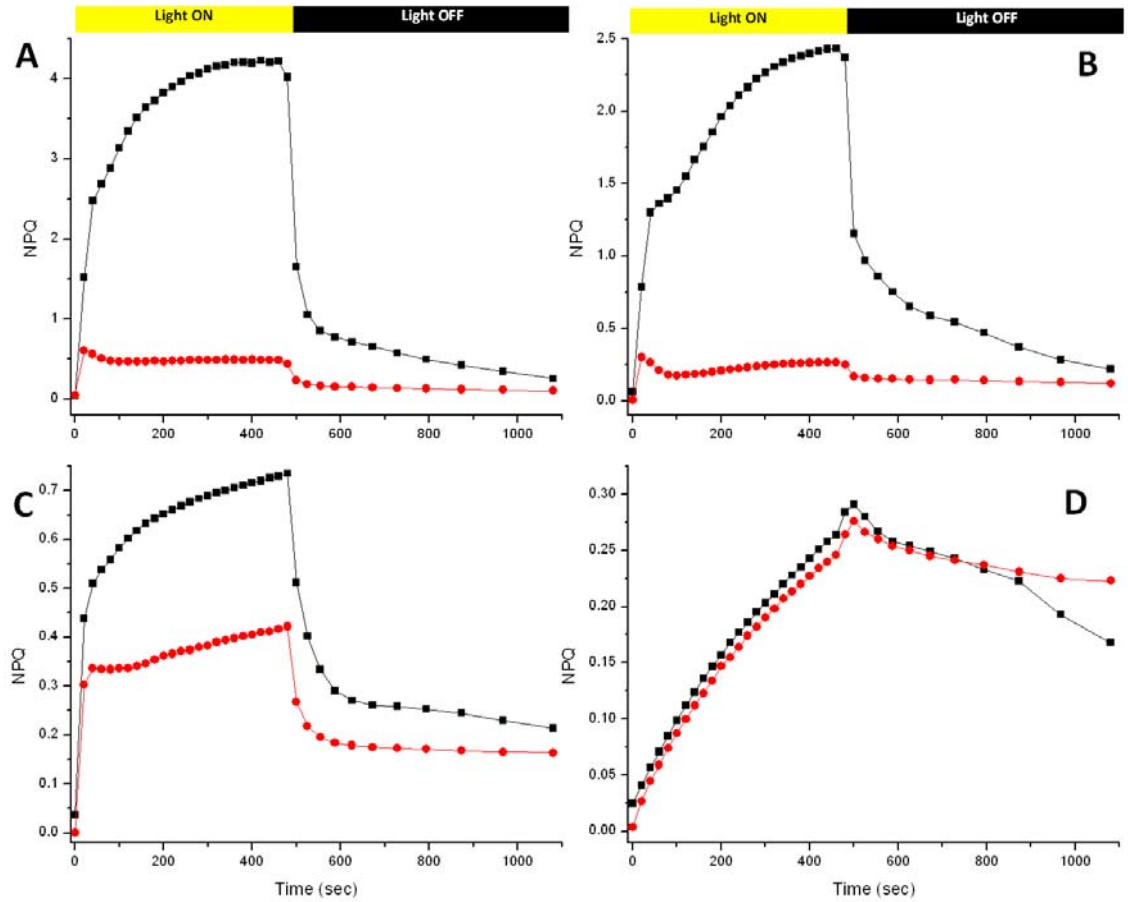

Figure 3. NPQ kinetics of mutants treated with DTT, a VDE inhibitor which is impairing the zeaxanthin synthesis. WT, *psbs* KO, *lhcsr1lhcsr2* KO and the triple KO (*psbs lhcsr1lhcsr2* KO) mutant are shown respectively in A, B, C and D. Samples were either treated with buffer alone (black squares) and DTT (red circles). The data reported for WT are the same of figure 1A. Note that the y axys scale is different in each graph, decreasing from A to D.

As in WT (figure 3A), in both *lhcsr1lhcsr2* KO and *psbs* KO residual quenching is present and identifiable as qE type. DTT effect is strong in LHCSR depleted mosses (*lhcsr1lhcsr2* KO is shown in fig. 3C), where NPQ is only PSBS dependent, as in *Arabidopsis* plants. This was expected as the NPQ activation mechanism is the same. However, DTT influence is strong in *psbs* KO plants as well, where instead NPQ is only LHCSR dependent (figure 3B). These results suggest that zeaxanthin synthesis is important for increasing both LHCSR and PSBS activity in energy dissipation. Interestingly, in the triple mutant where both PSBS and LHCSR are absent, we observed no phenotype from the DTT treatment (figure 3D), suggesting that in their absence zeaxanthin is not sufficient alone to activate any NPQ, at least in this timescale. Furthermore this result also suggest that the slow quenching observe in triple mutants is likely due to photoinhibition and not to the slowly activated zeaxanthin dependent NPQ component identified in *Arabidopsis* (Dall'Osto *et al.*, 2005; Nilkens *et al.*, 2010).

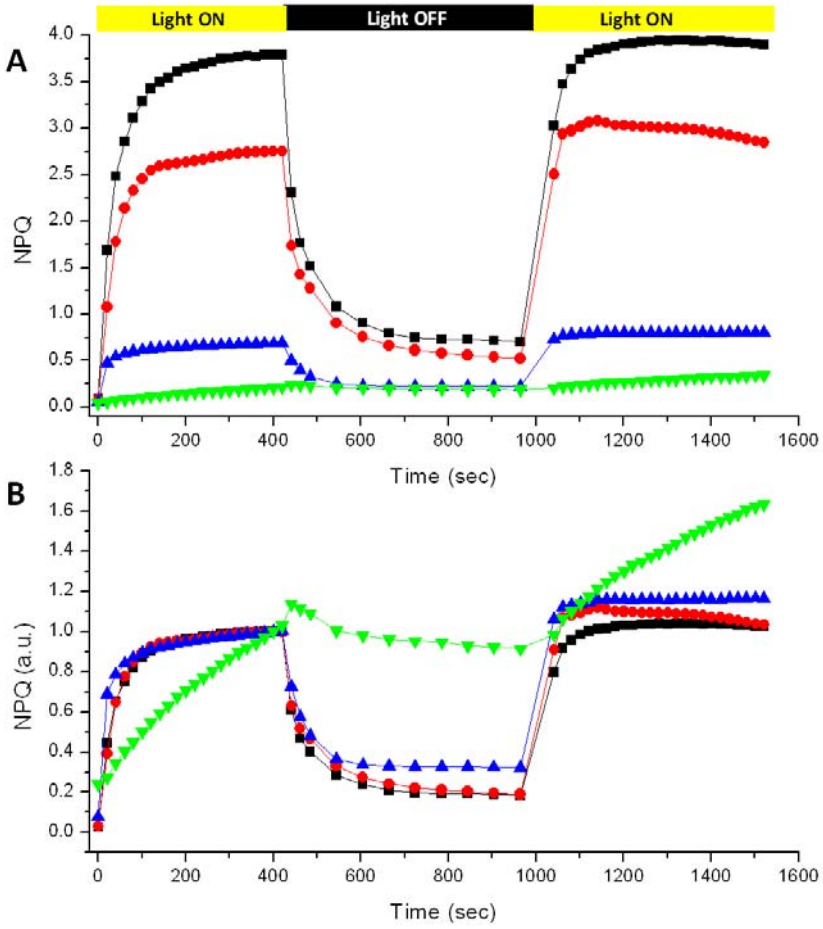

Figure 4. Zeaxanthin effect on NPQ measured by a double NPQ kinetics. A) Two NPQ kinetics were performed in rapid succession, separated by 10 minutes of dark relaxation, as in figure 2. WT, *psbs* KO, *lhcsr1lhcsr2* KO, and the triple *psbs lhcsr1lhcsr2* KO are shown in black squares, red circles, blue triangles and green triangles, respectively. B) kinetics shown in A were normalized to the maximal value reached at the end of the first light period. All lines except the triple KO show a similar kinetic irrespective from NPQ value reached.

We also analyzed double kinetics of these KO mutants grown in control condition. As in WT, in KO mutants we observed an acceleration in NPQ induction in the second kinetics (figure 4A). Interestingly, in all genotypes but the triple mutant we observed a very similar effect, irrespectively from the maximum NPQ level (Figure 4B), confirming the previous suggestion that zeaxanthin has a similar effect in enhancing both LHCSR and PSBS dependent NPQ. These results also confirm that in the absence of both PSBS and LHCSR zeaxanthin is not inducing any fast NPQ response.

Physcomitrella vde KO (npq1)

To further confirm the results obtained so far, we also produced *vde* KO mosses. As in the case of *psbs* and *lhcsr* KO, the targeting constructs was designed to substitute the entire coding sequence with selection cassette. Transformation and selection of resistant colonies were performed as in (Alboresi *et al.*, 2010) (chapter 2). Resistant lines were screened for the insertion of transgene in the correct locus, for the absence of VDE polypeptide in protein extracts and for their inability to accumulate zeaxanthin. Two independent lines were retained for further phenotype characterization (selected *vde* KO lines were kindly provided by Alessandro Alboresi, University of Verona). It is worth mentioning that the two selected lines are the results of independent transformations with plasmids carrying different selection cassette (Hygromycin and Zeocin resistance cassettes), as a further confirmation that the resulting phenotype is really due to the depletion of VDE protein and it is not caused by other possible secondary effects.

When analyzed for their capacity of inducing NPQ, these two lines shows a similar NPQ activation, which is strongly reduced compared to WT samples (figure 5). Interestingly, this phenotype is also very close to the effect of DTT treatment, as in the case of *Arabidopsis*. This confirms that the results obtained by DTT treatment are probably due to specific effects on NPQ induction and not to side effects of the inhibitor. We are now currently generating *vde* KO mosses also in *lhcsr1lhcsr2* KO and *psbs* KO backgrounds, to confirm the result previously shown and obtain analyses in a more physiological condition compared to DTT treatment.

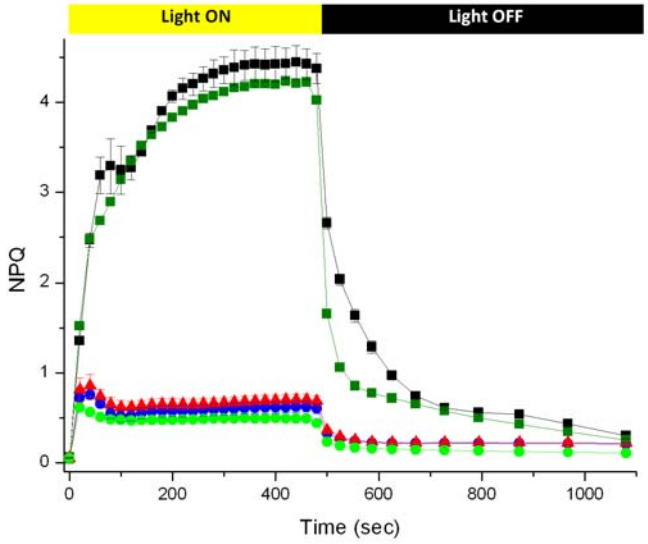

Figure 5. *Physcomitrella vde* KO mutants (*npq1*). NPQ kinetics of WT mosses (black) and *vde* KO mutant lines (Hyg^R and Zeo^R) shown in blue and red, respectively. Control and DTT treated mosses of figure 1A are reported as a comparison in dark green and bright green respectively.

Insights on NPQ mechanism: role of zeaxanthin

Zeaxanthin is known to be involved in NPQ activation and in particular in enhancing its intensity in plants (Niyogi *et al.*, 1997b; Holt *et al.*, 2005). Here we showed that zeaxanthin in *P. patens* is indeed fundamental in both PSBS and LHCSR activity and thus NPQ is strongly affected when VDE is inhibited by DTT. However, it should be remarked that in the absence of zeaxanthin a small but clear NPQ is measurable, suggesting that zeaxanthin is important but not indispensable for PSBS and LHCSR activity (figures 1, 3 and 5). This residual NPQ can also be recognized as qE from its fast activation and deactivation (figures 1, 3 and 5). NPQ kinetics in figures 2 and 4 confirm this role of zeaxanthin: here by repeating two NPQ kinetics we observe that the synthesis in the first curve is accelerating the second kinetic. However, normalization of these curves also showed that zeaxanthin effect on LHCSR and PSBS dependent NPQ is similar since the second kinetics is very similar in both cases, WT and all available mutants but the triple KO. While PSBS and LHCSR are active also in the absence of zeaxanthin the opposite is not true: when LHCSR and PSBS are both absent (triple KO) there is no effect of zeaxanthin on NPQ induction, at least in the fast components activated within the 8 minutes of illumination.

In addition, the DTT treatment phenotype of HL grown mosses (figures 1D and 2), which present pre-existing zeaxanthin accumulated during growth under strong light regimes, suggests that this pre-existing zeaxanthin is only marginally involved in the induction of qE, and that new zeaxanthin molecules need to be synthesized for the maximal induction of NPQ.

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CHAPTER 7

EVIDENCES OF EVOLUTION OF PSBS DEPENDENT NPQ IN LATE STREPTOPHYTA ALGAE

EVIDENCES OF EVOLUTION OF PSBS DEPENDENT NPQ IN LATE STREPTOPHYTA ALGAE

ABSTRACT

In previous chapters we evidenced how during plants evolution it emerged a new NPQ mechanism dependent on PSBS, first in addition and then substituting the one dependent from LHCSR, commonly present in algae. In order to investigate in more detail when this PSBS dependent mechanism evolved, we analyzed algae belonging to the Streptophyta lineage. These are the green algae which diverged later from the land plants ancestors. Among them we found that species belonging to the groups Charales and Coleochetales, which were the latest to diverge from plants, also showed evidences of a PSBS dependent NPQ. On the contrary, species which diverged earlier, as the Klebsomidiales, showed dependence on LHCSR for their NPQ.

INTRODUCTION

Algae are an heterogeneous group of organisms living in different habitats, going from green algae, red algae and glaucophyte to heterokontophyte (as diatoms) (Rodriguez-Ezpeleta *et al.*, 2005; Lee, 2008; Archibald, 2009). These organisms also display a surprising diversity in morphology, cell architecture, life histories, reproduction and biochemistry (Lewis & McCourt, 2004; Lee, 2008; Becker & Marin, 2009; Archibald, 2009). As example, algae include microscopic organisms as well as meter large ones, with different thallus organization, sessile or motile cells, filaments, colonies and species with various levels of tissue organization (Lewis & McCourt, 2004; Lee, 2008; Archibald, 2009). *Viridiplantae* is a monophyletic clade which comprises all green algae and plants. This group of organisms early split into two evolutionary lineages: Chlorophyta and Streptophyta, with the latter originating land plants ((Waters, 2003; McCourt *et al.*, 2004; Lewis & McCourt, 2004; Bowman *et al.*, 2007; Becker & Marin, 2009); see also figure 1). This separation occurred early, about 725-1200 MY ago depending on the different estimations (Yoon *et al.*, 2004; Hedges *et al.*, 2004; Zimmer *et al.*, 2007). The Streptophyta/Chlorophyta divergence was suggested to be correlated with a remarkably conservative preference for freshwater/marine habitats (Becker & Marin, 2009). The adaptation of some Streptophyte algae to freshwater, in fact, allowed the gradual colonization first of moist habitats in the proximity of water, and ultimately the colonization of dry land (Becker & Marin, 2009). Here the first land Streptophyte were

exploiting an entirely new environment, with very low competition with other photosynthetic organisms, probably with the exception of cyanobacteria and fungi (Becker & Marin, 2009). This allowed the explosive radiation of land plants, which adapted their morphology, physiology and reproduction to land life (Becker & Marin, 2009). Also some Chlorophyta species later developed the ability to live in freshwater and colonized some terrestrial environment. In this case, however, these species faced the opposite situation, with a strong competition with the already adapted land plants. It is therefore not surprising that no second lineage of land plants evolved successfully after the one raised from Streptophyta (Becker & Marin, 2009). On the other side, the existent Streptophyte algae display a low diversity compared to Chlorophyta, with only around one hundred genera for the former while several hundreds have been described for the latter (McCourt *et al.*, 2004; Becker & Marin, 2009). Six morphologically distinct groups have been described for Streptophyte algae: the flagellate Mesostigmatales, sarcinoid Chlorokybales, filamentous (unbranched) Klebsormidiales, Zygnematales (characterized by sexual reproduction by conjugation and the total absence of flagellate cells) and finally the two morphologically most complex groups, Coleochaetales and Charales. These two groups are both characterized by a true multicellular organization (with plasmodesmata) of parenchyma-like thalli or branched filaments with apical growth and oogamous sexual reproduction (McCourt *et al.*, 2004; Becker & Marin, 2009). A schematic tree of these algae is reported in figure 1 (adapted from (Waters, 2003)). It should be underlined that the precise phylogenetic relationship in this tree is still debated, in particular concerning the identification of the closest sister group of land plants. In fact, while most works indentify Charales (Karol *et al.*, 2001; McCourt *et al.*, 2004) as the most probable sister group of land plants, others suggest Coleochaetales (Petersen *et al.*, 2003) or both Charales and Coleochaetales as the sister taxon to embryophytes (as reviewed in (Waters, 2003; McCourt *et al.*, 2004; Becker & Marin, 2009)).

As detailed explained in the previous chapters, photosynthetic organisms evolved different mechanisms for photoprotection to face a variable environment where light can change from being limiting to in excess (Niyogi, 2000; Szabo *et al.*, 2005; Eberhard *et al.*, 2008; Li *et al.*, 2009). Land colonization also represented new challenges with its different physic-chemical conditions which required adaptation of pre-existing mechanisms (Waters, 2003).

A major photoprotection mechanism is Non Photochemical Quenching (NPQ), which leads to the dissipation as heat of excess energy absorbed. As already discussed in the previous chapters, NPQ activation depends on the presence of PSBS protein in vascular plants (Li *et al.*, 2000), while in the Chlorophyta *Chlamydomonas reinhardtii* (Peers *et al.*,

2009) and in some diatoms (Zhu & Green, 2010; Bailleul *et al.*, 2010) it requires a distinct Lhc-like protein, called LHCSR (or Li818). In chapters 2-4 we demonstrated that in the moss *Physcomitrella patens* NPQ activation relies on both LHCSR and PSBS (Alboresi *et al.*, 2010), and that these two mechanisms are active independently. All available data thus suggest a picture where algae and plants have different requirement for NPQ activation, the former dependent on LHCSR and the latter on PSBS. The two mechanisms overlapped during evolution, as the example of *Physcomitrella patens* suggests.
In this chapter we analyzed some species of Streptophyta algae (Charophyte) to verify when, during evolution, it was first evolved a PSBS dependent NPQ. Our results suggest that NPQ is dependent on LHCSR in the species belonging to Klebsormidiales (*Klebsormidium flaccidum* and *Interfilum terricolum*), while we detected PSBS in the species belonging to taxa believed to be the closest to land plants, Coleochetales and Charales (*Coleochaete scutata* and *Chara corallina*). This work thus suggests that during streptophyta evolution a PSBS dependent NPQ evolved in some streptohyta algae, close to land plants.

MATERIALS AND METHODS

Plant material. Culture of *Klebsormidium flaccidum, Interfilum terricolum, Coleochaete scutata* were obtained by SAG (Sammlung von Algenkulturen Göttingen, Culture Collection of Algae; SAG strain number 106.80 (*Kf*),2100 (*It*) 110.80M (*Cs*)). *Chara corallina* instead was kindly provided by Prof. Anna Moroni (Università Statale di Milano). *Kf* and *Cs* were grown on BG11 medium, while *It* cultures were propagated on Bold´s Basal Medium (BBM) with vitamins (BBM + V). Both media were solidified with 1% plant agar (Duchefa Biochimie). *Kf*, *It*, *Cs* cultures were propagated under sterile conditions on 9 cm Petri dishes and placed in a growth chamber under controlled conditions: 24°C and continuous light of 25-40 µE m^{-2} s^{-1}, and all analyses were performed on 5-weeks-old culture. For *Chara corallina* growth we used a plastic container of about 30x20x15 cm, filled with earth/sand covered with water. Tissues developed from internodal cells in about one month. The container was placed at room temperature near a window, with about 10 µE m^{-2} s^{-1} light intensity and natural photoperiod. All analyses were done after about 2 months of growth.

NPQ measurements. In vivo chlorophyll fluorescence of *Klebsormidium flaccidum, Interfilum terricolum, Coleochaete scutata* and *Chara corallina* was measured at room temperature with Dual PAM-100 fluorometer (Walz), using a saturating light at 6000 µE

m^{-2} s^{-1} and a light curve protocol of 20 steps with increasing actinic lights in the range 6-2000 µE m^{-2} s^{-1}. Before measurements, samples were dark-adapted for 40 minutes at room temperature. The parameter NPQ was calculated as (Fm-Fm')/Fm' (Demmig-Adams *et al.*, 1996).

Total protein extraction, SDS-PAGE and Western Blotting analyses. Total proteins extracts of the algae analyzed were obtained by mortar grinding of samples frozen with liquid nitrogen. Powder was then solubilized in Glicerol 30%, Tris 125mM pH 6.8, SDS 9%, DTT 0.1 M. Protein extracts were separated by SDS-PAGE and transferred onto a nitro-cellulose membrane (Pall Corporation) using a blot system from Amersham. Immunoblotting analysis were performed with home-made polyclonal antibodies for PSBS (1:500 in 0.01%Tween/TBS [TTBS]) and LHCSR (1:5000 in TTBS). For immunoblotting experiment with the saturation of the primary antibodies, the anti-PSBS/anti-LHCSR solution in TTBS was incubated with the recombinant PSBS or LHCSR protein, respectively, for 1 h before the incubation with the membrane. Different protein concentration were tested: 0.04, 0.4 or 4 µg protein/ml antibody in TTBS. The recombinant proteins used for the saturation were obtained from the expression in BL21 *E. coli* culture. Expression vectors (pQE50His with the coding sequence of barley [*Hordeum vulgare*] PSBS and pETMHis with the one of *Chlamydomonas* LHCSR3) were kindly provided by Dott. G. Bonente (University of Verona) (Bonente *et al.*, 2008a; Bonente *et al.*, 2011). PSBS was purified by affinity chromatography, while LHCSR was purified as inclusion bodies from *E. coli*.

RESULTS & DISCUSSION

All analyzed algae are able to induce an NPQ response.

In order to analyze NPQ dependence on LHCSR and/or PSBS in Streptophyta algae we selected four species, belonging to different groups within the Charophyte. In particular, we analyzed *klebsormidium flaccidum* and *Interfilum terricolum*, which belongs to Klebsormidiales, and *Coleochaete scutata* and *Chara corallina*, which are part of Coleochaetales and Charales, respectively. These species were selected as representative of their groups because they were available in public collections or from colleagues.

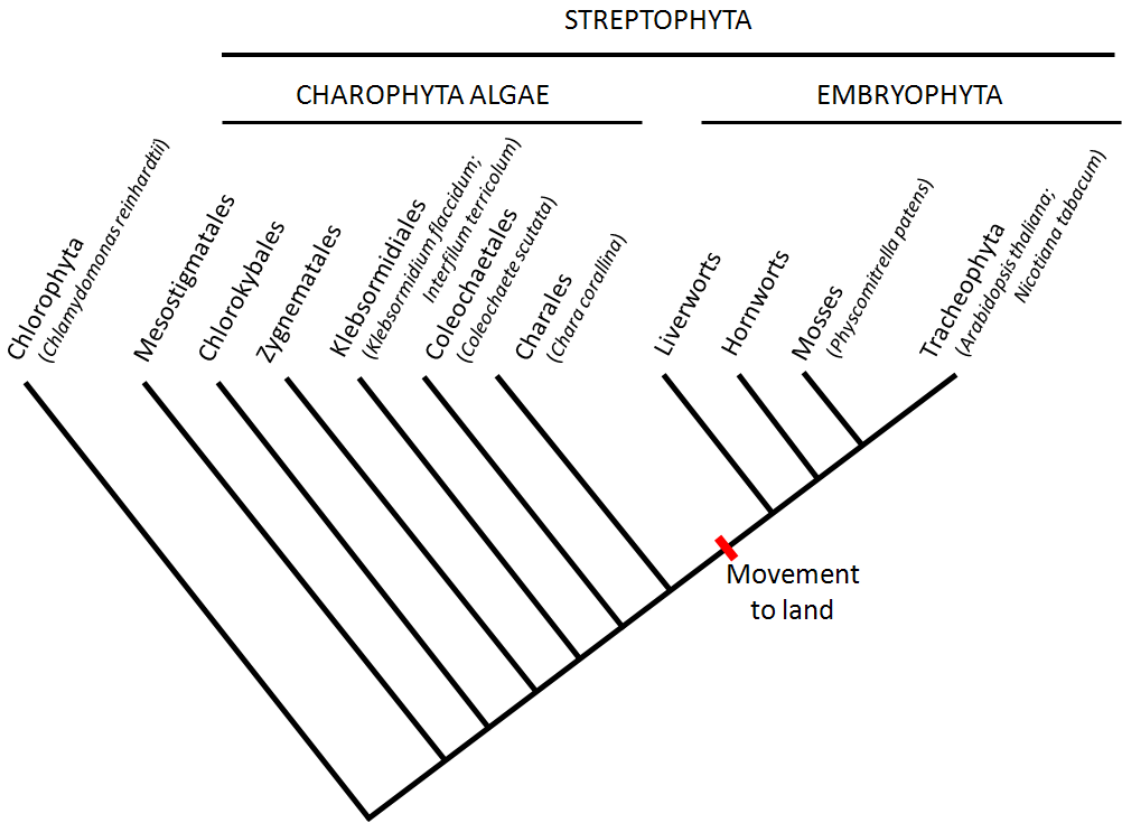

Figure 1. Phylogenetic tree in the Chlorophyta/Streptophyta lineage (V*iridiplantae*)
The tree depicts the ancient split between Chlorophyta and Streptophyta lineages and one of the current knowledge of Streptopyte phylogenetic relationship, with Charales as the sister group of land plants and liverworts as the most basal land plants (adapted from (Waters, 2003)). However, the identification of both the sister group to land plants and the most basal land plant group is still debated (as reviewed in (Waters, 2003; McCourt *et al.*, 2004; Becker & Marin, 2009)). The organisms used in this work as well as some model organisms to study NPQ process are shown.

As shown in figure 1, Klebsormidiales branch is suggested from many phylogenic studies to have a more basal position in the Streptophyte clade, while Coleochaetales and Charales groups are recognized as the closest to plants. Consistently, the latter are the most complex and they characterized by a true multicellular organization. The identification of either Coleochaetales or Charales as the true sister group of land plants is still debated, with different phylogenetic analysis providing different results (Waters, 2003; McCourt *et al.*, 2004; Becker & Marin, 2009).

First of all we verified if these algae were capable of inducing an NPQ response by monitoring chlorophyll fluorescence *in vivo*. Figure 2 reports the NPQ dependence from actinic light intensity and shows that all species respond to the increasing illumination by activating an NPQ response. *C. corallina* induced very rapidly a NPQ which however reaches saturation with a very low light intensity. This behavior is in part attributable to the fact that this species was grown at very low irradiation. In *K. flaccidum* as well NPQ reaches saturation at relatively low levels. *C. scutata* shows instead higher NPQ with

respect to *Chara* and *Klebsormidium*. The last species analyzed, *I. terricolum*, present a peculiar NPQ behavior, and values do not reached saturation but seems to increase further by increasing light. Most likely here other phenomena like photoinhibition or state transition are influencing fluorescence kinetics.

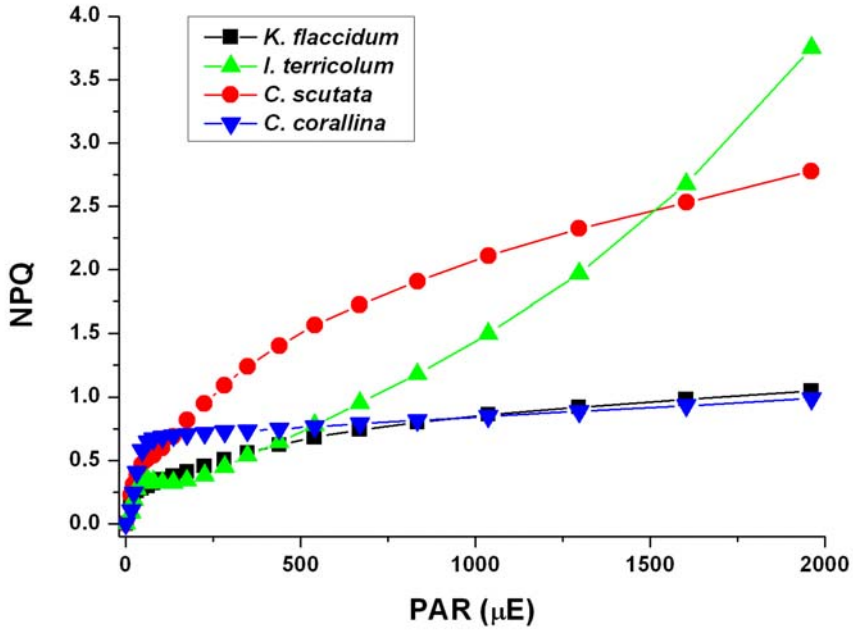

Figure 2. NPQ in the Streptophyte algae analyzed. Light curve protocols (20 steps of 60 seconds each, with actinic light increasing from 6 μE m^{-2} s^{-1} to 2000 μE m^{-2} s^{-1}) where employed to analyzed NPQ induction capacity of *Klebsormidium flaccidum*, *Interfilum terricolum*, *Coleochaete scutata* and *Chara corallina*, shown respectively in black, green red and blue.

These data show that all species are able to induce an NPQ response, with different kinetics and different final values reached. This is consistent with previous analyses (some examples are reported in (Masojidek *et al.*, 1999; Garcia-Mendoza *et al.*, 2002; Bonente *et al.*, 2008b; Grouneva *et al.*, 2009; Thaipratum *et al.*, 2009)) which showed a highly variable NPQ in different algae species, which is likely attributable to the species adaptation to their ecological niche. No particular correlation between NPQ levels and evolutionary position was observed in previous works (Bonente *et al.*, 2008b), as also confirmed here.

NPQ relies on PSBS or LHCSR in the various species analyzed

Once established that our organisms are able to activate NPQ, we tested these species for the presence of LHCSR and /or PSBS to identify which protein was responsible for this capacity. Total protein extracts were separated by SDS-PAGE and analyzed by immunobloting using polyclonal antibodies raised respectively against barley PSBS and *Chlamydomonas* LHCSR.

When tested with anti-PSBS antibody, we obtained a clear cross-reacting band in *Coleochaete scutata* and *Chara corallina* protein extracts, while for the other two species analyzed, *K. flaccidum* and *I. terricolum*, we found no cross-reactions (figure 3A). Bands detected in the algae samples have a molecular weight similar to plants PSBS, present in the same gel as controls (figure 3A). The antibody used is highly specific for PSBS polypeptides, with a clear immuno-detected band in many plant species (see also *N. tabacum* and *P. patens* samples in figure 3A). No additional bands were normally detected. To further confirm that the bands identified indeed corresponded to PSBS, we repeated the western blotting on *C. scutata* and *C. corallina* samples using a pre-saturation of PSBS antibody with the purified recombinant barley PSBS. The treatment of the polyclonal anti-PSBS antibody with the free recombinant PSBS led to the saturation of antibodies with high affinity for PSBS, thus blocking their ability to bind the polypeptides present on the membrane. As a consequence, the band/bands corresponding to the protein of interest will not be detected anymore on the membrane. Although this behavior works both for genuine and aspecific bands, we can expect the former to disappear first, being the one recognized with the strongest affinity by the antibody. In figure 3B this experiment is performed without any saturation (as control, panel a) and with different ratios of recombinant protein/antibodies (panels b and c): the bands identified as PSBS disappear completely with the antibody saturation with the low amount of recombinant protein (blue and black arrows, panel b, figure 3B) while other aspecific bands even increase their intensity upon saturation (red arrows, compare panels a, b and c, figure 3B), further confirming the hypothesis that the band detected in the control experiment can be attributed to PSBS protein (figure 3A, panel a in figure 3B). In the case of *Chara corallina* the presence of a PSBS protein has been confirmed also by mass spectrometry analysis (Bonente *et al.*, 2008b).

We can thus confidently identify PSBS expression in *Chara corallina* and *Coleochaete scutata*, while no cross-reacting band were present in the lanes with *K. falccidum* and *I. terricolum* extracts (fig. 3A). This was despite the fact that we loaded more protein extract in their case with respect to *Coleochaete* and *Chara*, where we instead obtained positive results. A possible alternative explanation is that the antibody failed to recognize the protein efficiently. However, this possibility seems unlikely since the same antibody recognized efficiently the recombinant *Chlamydomonas* PSBS expressed in bacteria with 30% of the affinity vs. plant isoform (Bonente *et al.*, 2008b). Since *Chlamydomonas* is far more distant organism we could confident believe that the antibody should be able to recognize also *Klebsormidium* e *Interfilum* PSBS proteins. This result thus suggests that PSBS is not accumulated in *K. falccidum* and *I. terricolum*.

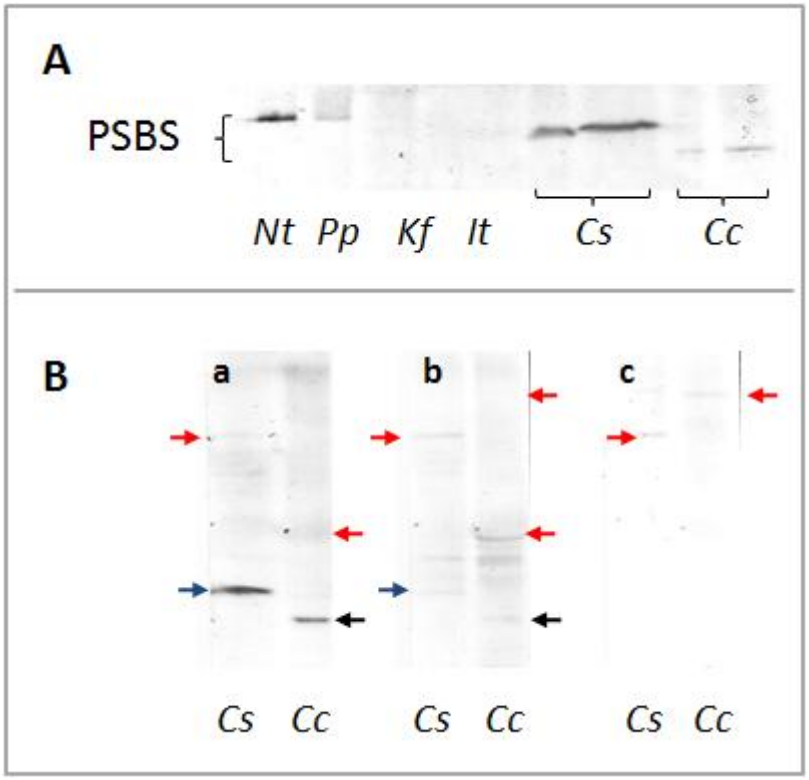

Figure 3. Western blotting against PSBS polypeptide in the Streptophyte algae.
A) Immunoblot with anti-PSBS antibody (1:500) on total extracts of the different algal species. *Kf*, *Klebsormidium flaccidum* (8 µg Chl); *It*, *Interfilum terricolum* (6 µg Chl); *Cs*, *Coleochaete scutata* (3 e 8 µg Chl); *Cc*, *Chara corallina* (3 e 8 µg Chl). *Cs* and *Cc* samples were loaded in different dilutions to verify signal linearity. *Nicotiana tabacum* (*Nt*, 2 µg Chl) and *Physcomitrella patens* (*Pp*, 4 µg Chl) thylakoids were loaded as controls. PSBS protein has slightly different molecular weight in various plants species.
B) Immunoblot for PSBS detection with the pre-saturation of the primary anti-PSBS antibody with recombinant barley PSBS. 3 µg Chl of *Coleochaete scutata* (*Cs*) and 7 µg Chl *Chara corallina* (*Cc*) protein extracts were loaded many times to test different concentration of recombinant PSBS for the saturation: panel a, anti-PSBS 1:500 TTBS without any saturation (control); panel b, saturation with 0.04 µg of PSBS recombinant protein /ml diluted antibody; panel c, saturation with 4 µg of PSBS recombinant protein /ml diluted antibody. Blue and black arrows: bands identified as PSBS in *Cs* and *Cc* respectively, as they disappear with the saturation of the antibody. Red arrows: aspecific band which instead increased their intensities upon the anti-PSBS saturation (see text for details).

We also analyzed the same protein extracts for the presence of LHCSR protein, the polypeptide with a key role in NPQ in algae as *Chlamydomonas* and diatoms. In all species we found many bands detected with anti-LHCSR antibody at the expected molecular weight (20-30 kDa) (figure 4A). This is due to the fact that the polyclonal antibody anti-LHCSR available recognizes other Lhc proteins in addition to LHCSR, because of a significant sequence similarity between these polypeptides. This was observed also in other species (*Chlamydomonas reinhardtii* and *Physcomitrella patens*). Therefore it is not possible to identify univocally one band as LHCSR. To distinguish LHCSR from aspecific Lhcs, we performed additional analyses using an anti-LHCSR primary antibody pre-saturated with the recombinant *Chlamydomonas* LHCSR3 purified from *E. coli*. As explained above in the case of PSBS, we can expect the genuine LHCSR

band/bands to disappear first, being the one/ones recognized with the strongest affinity by the antibody.

In figures 4B and 4C this experiment was performed for all the species using different ratios of recombinant protein/antibodies. Concerning *Klebsormidium* and *Interfilum* samples (figure 4B), with the largest concentration of recombinant protein (figure 4B, panel c) all bands present in the control (figure 4B, panel a) disappeared, thus providing no additional information. On the contrary, with the intermediate recombinant protein concentration (figure 4B, panel b) we could identify one band for *K. flaccidum* (blue arrow) and two for *I. terricolum* (black and red arrows) which disappeared while the others were still detectable. In *Interfilum* sample one of the two bands disappearing was weak also in the control sample (the one identified by red arrow) suggesting this might still be an aspecific band. These results, although they must be taken with some caution, suggest that LHCSR protein is present and expressed in *Klebsormidium* and *Interfilum*.

In the case of *Coloechaete scutata* and *Chara corallina*, instead, we obtained no conclusive results from these experiments (figure 4C), as the signals of the bands present in the controls (figure 4C, panel a) are affected in a similar manner after the saturation of the antibody (figure 4C, panels b and c). Only the band indicated by the red arrow in *C. scutata* sample disappeared, but it was weak also in the control and thus it could be an aspecific. The experimental condition used in the experiment presented in figure 4C will be further optimized testing different concentration of recombinant protein for the saturation to obtain clearer information about the presence of LHCSR in *C. scutata* and *C. corallina* extracts.

A possible explanation for LHCSR missed identification is that these cultures have been grown under dim illumination: although in some cases as in *Physcomitrella* (Alboresi *et al.*, 2010), in the diatom *Phaeodactylum tricornutum* (Bailleul *et al.*, 2010) and in *Klebsormidium* and *Interfilum* there is a constitutive expression of LHCSR, in others as *Chlamydomonas reinhardtii* (Peers *et al.*, 2009) and in the diatom *Thalassiosira pseudonana* (Zhu & Green, 2010) its expression has been shown to be strongly induced by high light. Thus, LHCSR accumulation can be dependent on the light irradiation also in these species, and we are currently testing other growth conditions for *Coleochaete scutata* and *Chara corallina* to verify this hypothesis. In fact, genes encoding for LHCSR has been found in all algae and also in mosses such as *P. patens* and *Tortula ruralis* (Koziol *et al.*, 2007; Alboresi *et al.*, 2008; Alboresi *et al.*, 2010; Engelken *et al.*, 2010). This gene thus was also present in the common ancestor between *Coleochaete scutata*, *Chara corallina* and plants and, unless it has been lost successively by these species, *LHCSR* gene should be present in their genome.

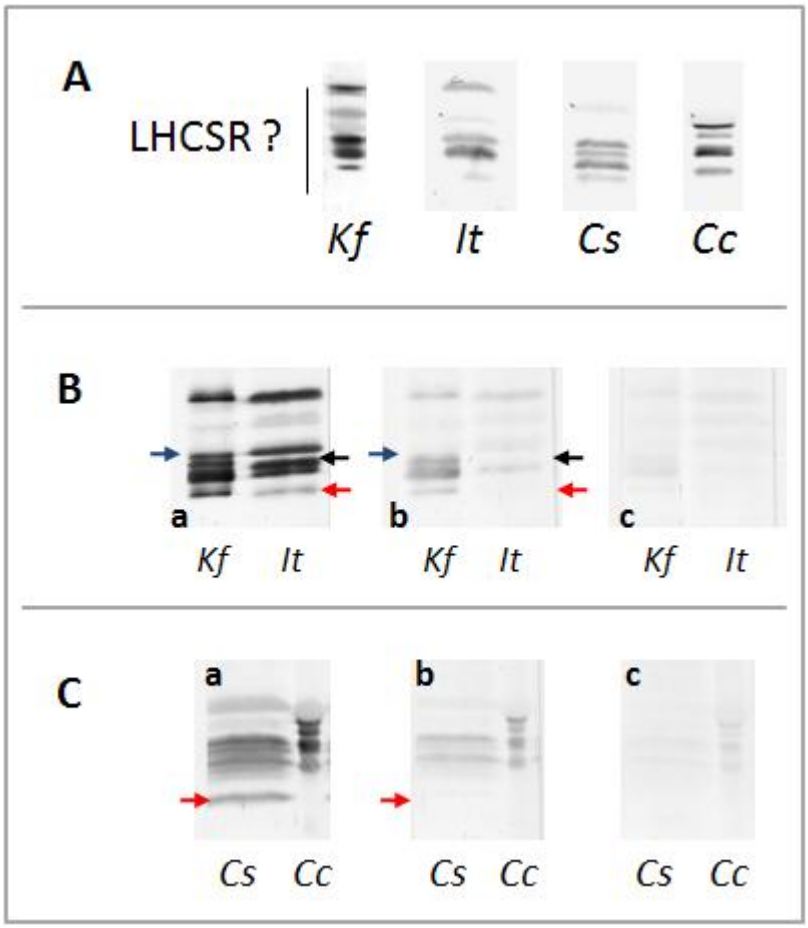

Figure 4. Western blotting against LHCSR polypeptide in the Streptophyte algae.
A) Immunoblot with anti-LHCSR antibody (1:5000) on total extracts of the different algal species. *Kf*, *Klebsormidium flaccidum* (3 µg Chl); *It*, *Interfilum terricolum* (2 µg Chl); *Cs*, *Coleochaete scutata* (2.5 µg Chl); *Cc*, *Chara corallina* (2 µg Chl). Bands in the range 20-30 kDa molecular weight are shown: LHCSR weight vary in different species, as *Physcomitrella patens* (23 kDa) and *Chlamydomonas reinhardtii* (29 kDa), and thus all these bands can be due to an LHCSR polypeptide.
B) Immunoblot for LHCSR detection with the pre-saturation of the primary anti-LHCSR antibody with recombinant LHCSR3 of *C. reinhardtii*. 2 µg Chl of *Klebsormidium flaccidum* (*Kf*) and *Interfilum terricolum* (*It*) protein extracts were loaded many times to test different concentration of LHCSR3 for the saturation: panel a, anti-LHCSR 1:5000 TTBS without any saturation (control); panel b, saturation with 0.04 µg recombinant protein /ml diluted antibody; panel c, saturation with 0.4 µg recombinant protein /ml diluted antibody. Blue and black arrows: bands identified as LHCSR isoforms in *Kf* and *It* respectively, as they disappear with the saturation of the antibody. Red arrows: band probable associated with an aspecific recognition of the antibody in *It* sample (see text for details).
C) Immunoblot for LHCSR detection with the pre-saturation of the primary anti-LHCSR with recombinant LHCSR3 of Chlamy. 2 µg Chl of *C. scutata* (*Cs*) and *C. corallina* (*Cc*) protein extracts were loaded many times to test different concentration of LHCSR3 for the saturation: panel a, anti-LHCSR 1:5000 TTBS without any saturation (control); panel b, saturation with 0.04 µg recombinant protein /ml diluted antibody; panel c, saturation with 0.4 µg recombinant protein /ml diluted antibody. Red arrows: band probable associated with an aspecific recognition of the antibody in *Cs* sample (see text for details).

Conclusions: evolution of PSBS dependent NPQ mechanism in the green lineage

In previous chapters we discussed in much detail how the study of the moss *Physcomitrella patens* provides information on the plants adaptation to land environment. To further investigate evolution from water to land we here investigated

also some Streptophyta algae, the species which diverged later from ancestors of present plants. Although we still miss complete genome sequences and tools for genetic manipulation, making difficult a complete analysis of these organisms, there is still the need for their study these group of organisms to investigate plants evolution (Becker & Marin, 2009).

Present data suggest that PSBS gene was likely present in the common ancestor of all *Viridiplantae*, since a PSBS gene is present in all plants as well as in Chlorophyta algae (eg. *Chlamydomonas*, *Ostreococcus*) (Koziol *et al.*, 2007; Alboresi *et al.*, 2008). Nevertheless, PSBS protein is fundamental for plants NPQ whereas most algae likely relies on LHCSR. Recent data from literature proved that LHCSR polypeptides are involved in NPQ in diatoms (Zhu & Green, 2010; Bailleul *et al.*, 2010), which are algae not belonging to the green lineage, as well as in the model Chlorophyta *C. reinhardtii* (Peers *et al.*, 2009), suggesting a widespread contribution of LHCSR in NPQ in many other algae. In the previous chapters we demonstrated that in the moss *Physcomitrella patens* these two mechanisms overlapped, with both PSBS and LHCSR active in inducing NPQ. We here focused our attention on some Streptophyte algae, which are the ones which diverged later from land plants, to obtain some clearer information on the evolution of NPQ from algae to plants.

The major result of this part of the work was the identification of PSBS protein in *Coleochaete scutata* and *Chara corallina*. In the same algae LHCSR, if present, is likely expressed at a low level, suggesting that in these cells NPQ is PSBS dependent. At the same time PSBS is absent, or expressed at a very low level, in *K. flaccidum* and *I. terricolum* where instead we were able to detect LHCSR. Although we cannot absolutely exclude PSBS presence we should remind that NPQ requires significant amount of protein accumulation (see chapter 4) and that very small protein amounts even if present are not able to induce a strong response and thus are functionally less significant. We can thus conclude that in these two latter organisms NPQ is mainly LHCSR dependent.

These data, together with the ones presented in previous chapters and all present literature, allow proposing a scheme for evolution of NPQ, as reported in figure 5. Diatoms, which are a group of algae originated from secondary endosymbiosis with a red alga and thus are not part of the green lineage, rely on LHCSR for their NPQ and do not present PSBS orthologs in their genomes (Zhu & Green, 2010; Engelken *et al.*, 2010; Bailleul *et al.*, 2010). LHCSR dependent NPQ is also widespread in many green organisms, being present in Chlorophyte as *Chlamydomonas*, as well as in Charophyte algae like *Klebsormidium* and *Interfilum* and mosses as *Physcomitrella* and *Tortula* (Peers *et al.*, 2009; Alboresi *et al.*, 2010). Upward in the phylogenetic tree, instead, LHCSR gene

was lost, being absent from vascular plants genomes (Koziol *et al.*, 2007; Engelken *et al.*, 2010). On the contrary, an ancient PSBS gene was likely present in the common ancestor of all green lineage (*Viridiplantae*), as it is present in the sequenced genomes of species belonging to *Viridiplantae* going from Chlorophyte to vascular plants (Koziol *et al.*, 2007; Engelken *et al.*, 2010). Nevertheless, this protein seem not expressed in the majority of algae as in Chlorophyta (Bonente *et al.*, 2008b) and early diverging groups of Charophyta. PSBS expression and its role in NPQ instead likely appeared first in algal groups closer to land plants in the evolution, Charales and Coleochaetales. PSBS dependent NPQ overlapped with LHCSR also in the first land plants, as the example of *Physcomitrella* suggests (Alboresi *et al.*, 2010), to become then the only protein involved in NPQ in vascular plants (Li *et al.*, 2000). The overlap of LHCSR and PSBS mechanisms was likely fundamental because, in the absence of any NPQ, algae/plants colonizing land environment would likely be counter-selected. This is proved by the photosensitivity of many mutants depleted in their NPQ capacity: it was demonstrated for *psbs* KO *Arabidopsis* plants (Kulheim *et al.*, 2002), *Chlamydomonas* cells depleted in LHCSR (Peers *et al.*, 2009), and it was shown in mosses by the strong light sensitive phenotype observed in *Physcomitrella psbs* and/or *lhcsr* KO mutants ((Alboresi *et al.*, 2010); Chapter 3). Thus, in correspondence to land colonization PSBS dependent NPQ evolved while LHCSR was still ensuring sufficient photoprotection.

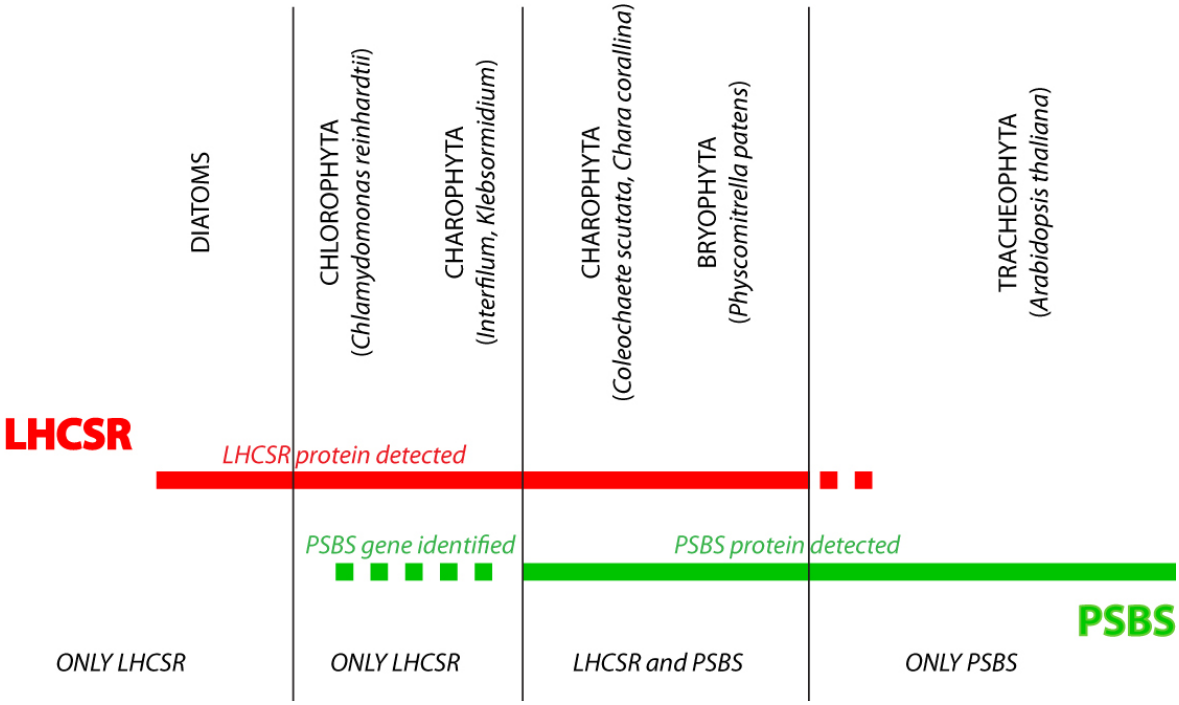

Figure 5. Evolution of NPQ mechanisms from algae to vascular plants.
Evolution of LHCSR- and PSBS-dependent NPQ in different species analyzed in literature or in this work. The presence of the LHCSR/PSBS protein during evolution is schematized as a red/green solid line respectively. When only the corresponding gene was identified we used a dashed line. The species were arranged on the basis of the available data (for the phylogenetic position of the species here reported, with the exception of diatoms, see figure 1):
- Diatoms: NPQ relies on LHCSR, PSBS being absent from their genomes (Zhu & Green, 2010; Engelken *et al.*, 2010; Bailleul *et al.*, 2010)
- Chlorophyte: in the model Chlorophyta *C. reinhardtii* NPQ relies on LHCSR (Peers *et al.*, 2009), PSBS ortholog is present but the protein not expressed (Bonente *et al.*, 2008b)
- Charophyte (early diverging groups as Klebsormidiales): we confidently identified LHCSR in these algae. According to the taxonomic position of these species, PSBS gene is likely present in their genomes as it is present also in Chlorophyte, and thus in the common ancestor of all green plants, but we did not identified PSBS polypeptide in these species. Thus, NPQ likely still relies on LHCSR in this group.
- Charophyte (Coleochaetales and Charales, the groups closer to land plants): we confidently identified PSBS in the protein extracts of *Coleochaete scutata* and *Chara corallina*. LHCSR likely is present as a gene, as it is found also in Bryophytes, but we obtained no conclusive results from western blot analysis for the presence of LHCSR protein in these species. NPQ thus likely relies on PSBS in these organisms, while LHCSR expression, if present, is probably induced by more stressful growth conditions, as in *Chlamydomonas*.
- Bryophytes (*Physcomitrella patens*): both LHCSR and PSBS active in NPQ (Alboresi *et al.*, 2010). The two mechanisms clearly overlapped during evolution: PSBS dependent NPQ evolved in land plants while LHCSR dependent one still ensured sufficient photoprotection.
- Tracheophyta (*Arabidopsis thaliana*): only PSBS is present in the genome, and thus PSBS is the key protein for NPQ induction (Li *et al.*, 2000; Koziol *et al.*, 2007; Engelken *et al.*, 2010).

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CONCLUSIONS

CONCLUSIONS

The focus of present work was the analysis of the fast protection mechanism NPQ. To this aim we exploited mainly the moss *Physcomitrella patens* as a model organism. One key advantage of this organism was the ability of doing homologous recombination, allowing for the efficient generation of KO and over-expressing plants.
In particular we generated plants with altered content in PSBS and LHCSR, the key proteins for NPQ in vascular plant and algae respectively.
Main conclusions of this work were:

- *Physcomitrella* is the only known organism where both PSBS and LHCSR proteins are active in NPQ
- PSBS and LHCSR dependent mechanisms are active simultaneously and independently and likely have different molecular mechanism
- The regulation of the accumulation of both polypeptides modulates NPQ amplitude and this is exploited by plants to regulate photoprotection according to environmental conditions. PSBS and the two LHCSR isoforms (LHCSR1 and LHCSR2) are also differently regulated in several environmental conditions, thus ensuring an optimal photoprotection.
- NPQ is a fundamental process both for short and long term responses of *P. patens* to strong illumination. Mutants with impaired NPQ showed stronger photoinhibition upon exposure to HL, after a few hours of treatment as well as after several days.
- Zeaxanthin enhanced both PSBS and LHCSR dependent NPQ, but it is unable to induce any fast quenching if these proteins are absent.

These results, together with the analysis of some Streptophyta algae, allowed proposing an hypothesis for the NPQ adaptation during plants evolution. While algae relies on LHCSR for their NPQ, later plants evolved an independent mechanism for NPQ depending on PSBS. These two mechanisms overlapped during evolution before plants lost LHCSR dependent NPQ, typical of algae.

APPENDIX

During my PhD I took also part to some other projects, beside the main research topic of the characterization of NPQ in *Physcomitrella* presented in the previous chapters.

As briefly report in the introduction, the analyses of *Physcomitrella* antenna polypeptides done by Alboresi et al., 2008 lead to the identification of a new polypeptide which showed sequence similarities with Lhcb proteins, and thus it was called Lhcb9. The protein was later better biochemically characterized showing peculiar properties. In this work I focused my attention on the analysis of the accumulation of Lhcb9 protein *in vivo* in *Physcomitrella* plants. Hereafter is reported the abstract of the paper submitted describing Lhcb9 biochemical and spectroscopic characterization.

Furthermore, during these years I collaborated with Prof. Barbara Baldan lab (University of Padova), where I started studies on abiotic stress in an unicellular Chlorophyta (*Chlorella saccharophila*) during my master degree thesis. I focused in particular on the role of cytochrome *f* in the heat shock (HS)-induced PCD (programmed cell death) and on the effects of a combined stress (salt stress +HS). Abstracts of two papers originated from these projects are here also reported.

A RED SHIFTED ANTENNA PROTEIN ASSOCIATED TO PHOTOSYSTEM II IN *PHYSCOMITRELLA PATENS*

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ABSTRACT

Antenna systems of plants and green algae are made up of pigment-proteins belonging to the light-harvesting complex (LHC) multigene family. LHCs increase the light harvesting cross section of photosystem I and II and catalyze photoprotective reactions which prevent light induced damages in oxygenic environment. The genome of the moss *Physcomitrella patens* contains two genes encoding LHCb9, a new antenna protein which bears an overall sequence similarity to Photosystem II antenna proteins but carrying a specific motif typical of Photosystem I antenna proteins. This consists in the presence of an asparagine residue as a ligand for Chl 603 (A5) chromophore rather than a histidine, the common ligand in all other LHCb. Asparagine as Chl 603 (A5) ligand generate red-shifted spectral forms associated to Photosystem I rather than to Photosystem II, suggesting that in *Physcomitrella patens* the energy landscape of Photosystem II might be different with respect to that of most green algae and plants. In this work we show that the *in vitro* refolded LHCb9-pigment complexes carry a red-shifted fluorescence emission peak, different from all other known Photosystem II antenna proteins. By using a specific antibody, we localized LHCb9 within PSII supercomplexes in the thylakoid membranes. This is the first report of red-shifted spectral forms in a PSII antenna system, suggesting that this biophysical feature might have a special role either in optimization of light use efficiency or in photoprotection in the specific environmental conditions experienced by this moss.

This paper has been submitted

CHLORELLA SACCHAROPHILA CYTOCHROME *f* AND ITS INVOLVEMENT IN THE HEAT SHOCK RESPONSE

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ABSTRACT

Cytochrome *f* is an essential component of the major redox complex of the thylakoid membrane. Cloning and characterization are presented here of a novel partial cDNA (*ChspetA*) encoding cytochrome *f* in the psychrophile unicellular green alga *Chlorella saccharophila* and its involvement in the heat shock (HS) response pathway has been analysed. Semi-quantitative reverse transcriptase PCR analysis showed that *ChspetA* expression is up-regulated in heat-shocked cells and the protein profile of cytochrome *f* highlighted a release of cytochrome *f* into the cytosol depending on the time lapse from the HS. Evans Blue assay, analysis of chromatin condensation, and chloroplast alterations showed the induction of cell death in cell suspensions treated with cytosolic extracts from heat-shocked cells. This study identifies cytochrome *f* in *C. saccharophila* that seems to be involved in the HS-induced programmed cell death process. The data suggest that cytochrome *f* fulfils its role through a modulation of its transcription and translation levels, together with its intracellular localization. This work focuses on a possible role of cytochrome *f* into the programmed cell death-like process in a unicellular chlorophyte and suggests the existence of chloroplast-mediated programmed cell death machinery in an organism belonging to one of the primary lineages of photosynthetic eukaryotes.

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PROGRAMMED CELL DEATH AND ADAPTATION: TWO DIFFERENT TYPES OF ABIOTIC STRESS RESPONSE IN A UNICELLULAR CHLOROPHYTE.

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ABSTRACT

Eukaryotic microalgae are highly suitable biological indicators of environmental changes because they are exposed to extreme seasonal fluctuations. The biochemical and molecular targets and regulators of key proteins involved in the stress response in microalgae have yet to be elucidated. This study presents morphological and biochemical evidence of programmed cell death (PCD) in a low temperature strain of *Chlorella saccharophila* induced by exposure to NaCl stress. Morphological characteristics of PCD, including cell shrinkage, detachment of the plasma membrane from the cell wall, nuclear condensation and DNA fragmentation, were observed. Additionally, a significant production of H(2)O(2) and increase in caspase 3-like activity were detected. We demonstrated that singly applied environmental stresses such as warming or salt stress trigger a pathway of PCD. Intriguingly, the prior application of salt stress seems to reduce heat shock-induced cell death significantly, suggesting a combined effect which activates a defense mechanism in algal cells. These results suggest that *C. saccharophila* can undergo PCD under stress conditions, and that this PCD shares several features with metazoan PCD. Moreover, the simultaneous exposure of this unicellular chlorophyte to different abiotic stresses results in a tolerance mechanism.

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ABBREVIATIONS

^{1}Chl*: singlet Chl excited molecules
$^1O_2$: singlet oxygen
^{3}Chl: Chl triplets
Arabidopsis, A. thaliana, At: Arabidopsis thaliana;
Car, Carotenoid;
Chara, C. corallina, Cc: Chara corallina.
Chl, Chlorophyll;
Chlamydomonas, C. reinhardtii, Cr: Chlamydomonas reinhardtii.
Coleochaete, C. scutata, Cs: Coleochaete scutata;
Cyt, Cytochrome;
DCCD: dicyclohexylcarbodiimide
DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea;
DTT, dithiotreitol;
F_0, minimal fluorescence of dark adapted sample;
Fdx, ferredoxin;
Fm, maximal fluorescence of dark adapted sample
Fm', maximal fluorescence of light exposed sample
FNR, Ferredoxin $NADP^+$ reductase;
Fv/Fm, It is the ratio between variable fluorescence and maximal fluorescence of dark adapted sample
HR, homologous recombination;
hv, light energy.
Interfilum, I. terricolum, It: Interfilum terricolum;
IR, illegitimate recombination;
Klebsormidium, K. flaccidum, Kf: Klebsormidium flaccidum;
KO, knock-out;
Lhc, Light harvesting complex;
Lhca, antenna polypeptides of Photosystem I;
Lhcb, antenna polypeptides of Photosystem II;
LHCI, antenna system of Photosystem I;
LHCII, Major antenna complex of Photosystem II;
LHCSR, Lhc-like protein Stress Related (previously called Li818, Light Induced protein 818).
NHEJ, Non-Homologous End Joining;
NPQ, Non Photochemical Quenching;
OE, over-expression/expressor;
PAGE, polyacrylamide gel electrophoresis;
PC, plastocyanin;
PCR, polymerase chain reaction;
Physcomitrella, P. patens; Pp: Physcomitrella patens;
PQ/PQH_2, plastoquinone/plastoquinol;
PSBS photosystem II subunit S;
PSII (PSI): photosystem II (I);
qE, Energy-dependent component of NPQ;
qI, photo-Inhibitory quenching, component of NPQ;
qT, component of NPQ related to State transition
ROS, Reactive oxygen species;
RT, room temperature;
RuBisCO, ribulosio 1,5biphosphate carbossilase-oxygenase;

SDS, sodium-dodecyl-sulphate;
TBS, Tris buffered saline
TGR, targeted gene replacement;
VDE, Violaxanthin de-epoxidase;
viola, violaxanthin;
WB, western blot;
WT, wild type.
ZE, Zeaxanthin epoxidase
zea, zeaxanthin;
ΔpH: pH gradient

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