UNIVERSITÀ DEGLI STUDI DI VERONA - DIPARTIMENTO DI BIOTECNOLOGIE

SCUOLA DI DOTTORATO IN SCIENZE NATURALI ED INGEGNERISTICHE

CICLO XXXIII

BALANCING LIGHT EFFICIENTLY:

THE LONG ROAD TO A SUSTAINABLE FUTURE

S.S.D. BIO/04

Coordinator: Prof. Roberto Bassi

Tutor: Dr. Alberta Pinnola

PhD-student: Christo Schiphorst

Balancing light efficiently: The long road to a sustainable future
Christo Schiphorst – PhD thesis
Verona, 30 April 2021

Voor mijn opa, zonder wie ik nooit had kunnen beginnen.

Voor Manja, zonder wie ik nooit zou zijn begonnen en zonder wie ik het nooit af had kunnen maken.

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Summary

Photosynthetic organisms harvest light and convert energy into biomass through the process of photosynthesis. Antenna systems are responsible for light harvesting and transfer the energy towards the Reaction Centres (RCs). However, too much light can be damaging and therefore the excess energy is dissipated as heat through a process called Non-Photochemical Quenching (NPQ). NPQ is activated by decreased pH on the luminal side of the thylakoid membranes by either PSBS or LHCSR found in higher plants and green algae respectively. The need for this type of regulation can be explained based on the extreme variability of the photon flux received by photosynthetic organisms during the day: at sunrise light is limiting and fully used for fuelling electron transport (ET) from H_2O to CO_2 to form sugars. At midday, however, light can be too much and the singlet Chl excited states produced by photon absorption cannot be quenched by photochemistry fast enough, leading to intersystem crossing to create Chl triplet states, which react with O_2 and results in the synthesis of Reactive Oxygen Species (ROS). ROS accumulation causes photoinhibition of photosynthesis which drastically limits plant growth. Therefore, dissipating light when in excess and using even the last photon under low light is a difficult exercise which is essential to ensure the maximal growth rate. However, plants are more "interested" in surviving stress and reproducing than in growing big. Therefore, they have developed a hysteretic response to light: in order to avoid damage, plants over-regulate energy dissipation thus growing less than could be afforded under farming conditions where the abiotic stress is limited. This indicates that there is large room for engineering energy dissipation and increase crop production, as shown by seminal work.

Furthermore, besides changes in intensity plants experience changes in spectral composition as well. However, PSII and PSI have slightly different absorbance spectra and for an efficient linear electron flow between the two photosystems, it is essential that the excitations between the two photosystems are balanced for optimal photosynthetic efficiency. This is regulated by the so-called state transitions a shuttling of antenna proteins between PSII and PSI.

In Chapter 1 the differences and similarities between a variety of oxygenic photosynthetic organisms is reviewed. The focus lies on the different sets of antenna systems that evolved during the evolution and how the antenna systems that we currently find in plants and green algae have such an important role in photoprotection.

In Chapter 2, PSBS in *A. thaliana* has been replaced with LHCSR1 from the moss *P. patens*, an evolutionary intermediate both expressing functional PSBS and LHCSR. The transformed *A. thaliana* lines showed a partial recovery of NPQ. The partial recovery of NPQ was mainly caused by the reduced capacity to convert violaxanthin into zeaxanthin in *A. thaliana* in comparison with *P. patens*.

In chapter 3, several different *A. thaliana* lines lacking one or more PSII-antenna complexes were transformed with LHCSR1 form *P. patens* in order to identify a possible interaction partner of LHCSR1, where Lhcb5 (CP26) has been identified as the most likely interaction partner of LHCSR1.

In chapter 4, the transformed lines from Chapter 2 were grown in different fluctuating light conditions to see whether LHCSR1 could increase the biomass production. However, in all cases WT grew the same or better than the transformed lines. In specific cases the presence of LHCSR1 could partly improve growth in comparison to a line without PSBS.

In Chapter 5, we looked at the locations of interaction of LHCII with PSI. This is especially interesting since LHCII is the most important protein to induce NPQ, and does perform state-transitions, a process which is essential for an even energy distribution between the two photosystems and therefore necessary to grow properly. A second LHCII-PSI interaction site has been confirmed by looking at the energy transfer in isolated stroma membranes in State I or State II of WT and a mutant devoid of the PSI antenna. We show that the presence of the PSI-antenna (Lhca1-4) increase the rate of energy transfer from LHCII to PSI by 4 times and thus are essential for a proper binding of LHCII to PSI, besides the the PsaH/L/O site.

Introduction

Photosynthesis is the biological process where light energy is used to produce organic compounds, it is the driving force of live found on earth. However, there are many different types of photosynthesis besides, the most well-known, the one used by plants and algae. Even though photosynthesis is important and can be found all around us, a large part of the basic principles is still lacking. The understanding of photosynthesis, or the lack of it for that matter, is something that might become important in the near future due to the ever-increasing demand for food.

Photosynthesis can be divided in two main categories based on the pigments used, the best known and most widely used is the chlorophyll-based photosynthesis and the other type is the rhodopsin-based photosynthesis. The latter has never received a lot of attention due to its perceived unimportance. The rhodopsin-based photosynthesis functions by directly transporting ions over a membrane using a cis-trans isomerization of the rhodopsin molecule (Lanyi 2004). However, it is becoming clear that the rhodopsin-based photosynthesis is more common than previously assumed, especially since it seems that the major part of the marine bacteria possesses rhodopsin which can be used to completely sustain or, more likely, supplement these 'heterotrophic' marine bacteria in their energy requirements (Pinhassi et al. 2016; Gómez-Consarnau et al. 2017).

The other category, the chlorophyll-based photosynthesis, uses light to drive an electron transfer and can be further subdivided into oxygenic and anoxygenic photosynthesis. The most well-known type is the oxygenic photosynthesis and it is performed by trees, plants, mosses, algae and cyanobacteria to provide them in their energy requirements. The oxygenic photosynthesis uses water as an electron donor and therefore produces, as the name suggests, oxygen as a by-product.

The anoxygenic photosynthesis is specific for certain groups of bacteria which are highly diverse and can be found in a variety of environments, such as in sewage treatment ponds, the nodules of leguminous plants, below the chemocline of stratified lakes and even in thermophilic environments such as the alkaline hot springs of Yellowstone park (Fleischman and Kramer 1998; Kolber et al. 2001;

Bryant et al. 2007; Blankenship 2014). These anoxygenic bacteria do not use water as an electron donor, but are able to use a wide variety of other compounds such as hydrogen sulphide, thiosulfate or in some cases even hydrogen (Frigaard and Dahl 2008; Blankenship 2014). Since they do not use water as an electron donor, there is no production of oxygen, hence the name anoxygenic photosynthesis. The only bacteria able to perform the oxygenic photosynthesis are the cyanobacteria, also known as blue-green algae (despite the fact that they are not algae). They are one of the most widely spread photosynthetic organisms and can be found almost everywhere as long as there is light, from fresh or salt water to desserts and from the frozen planes of the Antarctic's to the hot springs in Yellowstone park (Blankenship 2014). Some strains of cyanobacteria are even able to switch between water and hydrogen sulphide as an electron donor and are thus able to switch between the oxygenic and anoxygenic photosynthesis (Padan 1979). Due to the widespread nature of cyanobacteria, it is not hard to imagine that the oxygenic photosynthesis that we find in the photosynthetic eukaryotes this day, such as plants and algae, arose from one or multiple endosymbiotic events, where a eukaryote incorporated a cyanobacterial ancestor (Margulis 1981; Keeling 2010; Blankenship 2010; Jensen and Leister 2014). The incorporated cyanobacteria became the plastids responsible for photosynthesis, such as cyanelles, rhodoplasts and chloroplasts, that we currently find in the glaucophytes, red and green algae, respectively (Keeling 2010; Jensen and Leister 2014). The plastids have undergone a lot of changes from the initial cyanobacteria, such as gene transfer to the nucleus and specialization of the light harvesting antenna to different environments (Jensen and Leister, 2014; Moreira et al., 2000).

Oxygenic photosynthesis

Even though the plastids and cyanobacteria have undergone a lot of changes during the evolution, the core function and mechanisms of the oxygenic photosynthesis stayed the same throughout evolution (Allen et al. 2011).

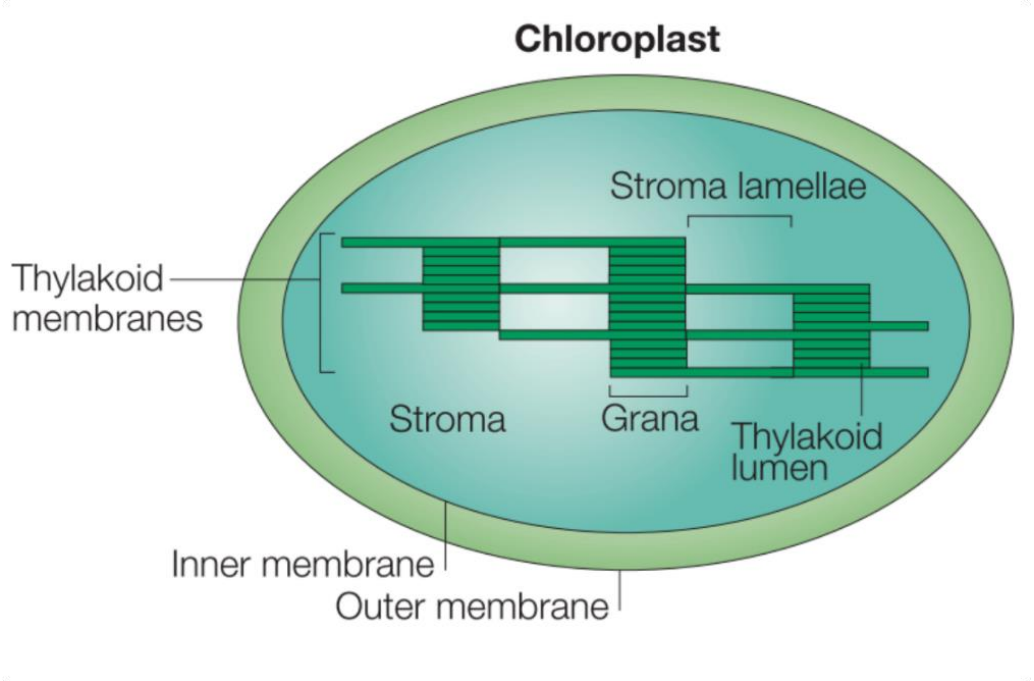

Figure 1 Schematic picture of a chloroplast

Chloroplasts have two outer membranes enveloping the stroma fluid which contains the thylakoid membranes. The thylakoid membranes in higher plants house the photosynthetic complexes in two different distinct regions. The grana are stacked circular disks harbouring PSII copmlexes while the stroma lamellae are the flat panels interconnecting the grana membranes that contain the PSI complexes. Figure from (Nelson and Ben-Shem 2004)

In plants and green algae, the oxygenic photosynthesis takes place in the chloroplasts. The chloroplasts have two outer membranes enveloping a soluble phase called the stroma, containing a third membrane set called the thylakoid membranes, see Figure 1. In the stroma the Calvin-Benson cycle fixes carbon into carbohydrates using NADPH and ATP. The Calvin-Benson cycle is sometimes also referred to as the ‘dark reactions’, even though it does also take place during the day, but since it can technically function without light as long as NADPH and ATP are present or if these are provided in another way. In normal conditions the

NADPH is produced by the linear electron transport in the thylakoid membranes and ATP by the proton gradient that is created during this electron transport. These reactions are sometimes referred to as 'light reactions', since these can only take place when there are photons present to drive the electron transport.

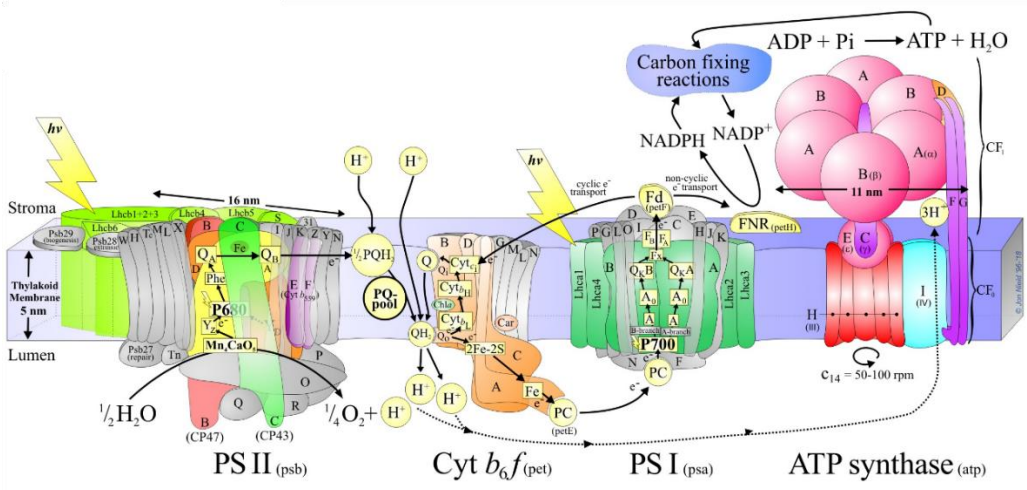

Figure 2 Schematic overview of the linear and cyclic electron transport

Overview of the arrangement of the protein complexes in the thylakoid membranes responsible for the oxygenic photosynthesis in green algae and plants. Figure courtesy of Jon Nield, Queen Mary, University of London, UK.

The thylakoid membranes located in the soluble stroma harvest the light and house the electron transport chains. The thylakoid membranes have a very distinctive structure with stacks of membranes, called the grana which are connected by membranes called stroma lamellae, see Figure 1. The main protein components of the thylakoids are the small and mobile electron carriers plastoquinone (PQ) and plastocyanin (PC) that transport electrons between the much bigger protein complexes Photosystem II (PSII), Cytochrome b_6f (Cyt b_6f) and Photosystem I (PSI). The electron transport is coupled to a proton displacement over the thylakoid membranes, creating a proton motive force (pmf) which is used to create ATP by ATP synthase (ATPase), see Figure 2.

The protein complexes are not homogeneously distributed in the thylakoid membranes. PSII is mainly located in the grana, while PSI and ATPase are located

in the stroma lamellae, Cyt b_6f is found in both grana and stroma lamellae (Vallon et al. 1991), see Figure 3.

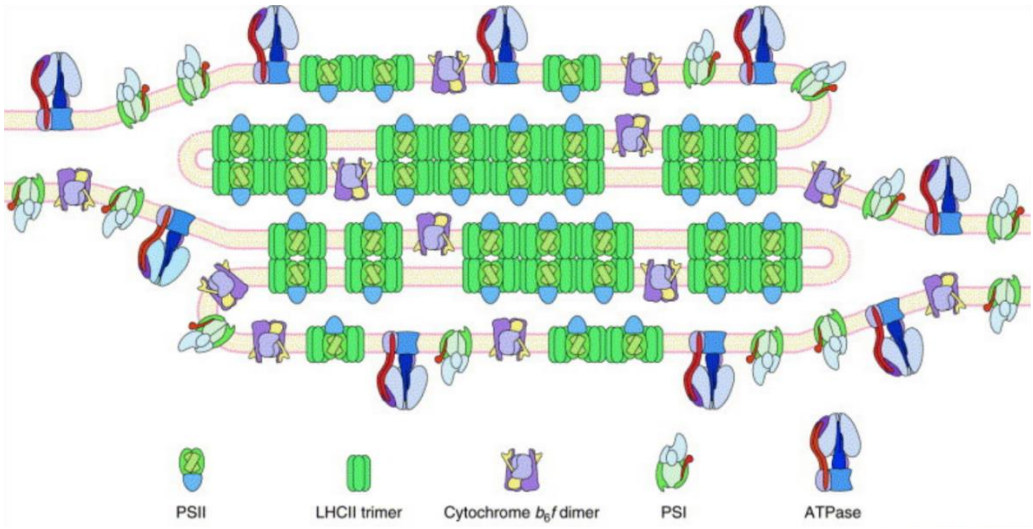

Figure 3 Overview of the lateral distribution of the different complexes in the thylakoids

The thylakoids membranes do not show a homogeneous distribution of the different reaction centres, instead PSI is mainly found in the stroma while PSII is found in the grana. Cyt b_6f is found in both thylakoid domains. Figure is from (Allen and Forsberg 2001)

The two photosystems, PSII and PSI, contain the reaction centres (RCs) that use light to excite electrons. Although the two RCs can function by themselves, they are surrounded by antenna complexes to increase the efficiency of light harvesting, Light Harvesting Complex II for PSII and Light Harvesting Complex I for PSI. The antenna complexes funnel the energy towards the photosynthetic RCs, which come in two sorts, depending on the type of electron acceptors: a quinone, in type II (found in PSII), and an Iron-Sulphur cluster in type I (found in PSI) (Nitschke and William Rutherford 1991).

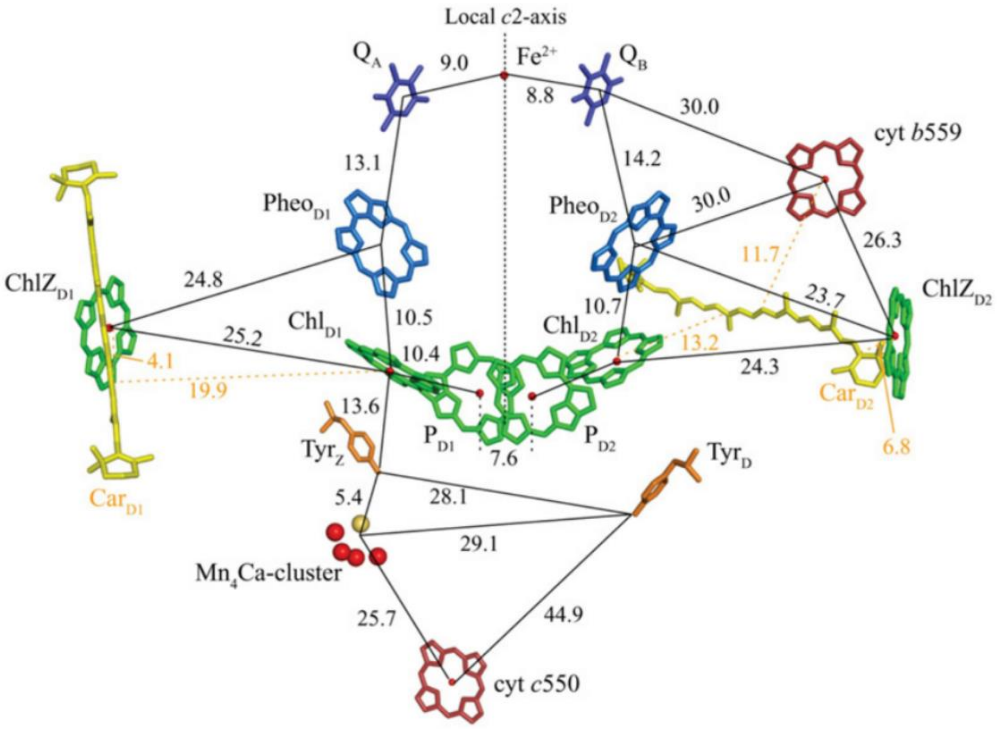

Figure 4 Structure of the cofactors in the reaction centre of PSII

The different cofactors are indicated together with the distances in Å. The Chlorophylls from the special pair (P680) are indicated by P with their respective core subunit (D1 or D2). Pheophytin as Pheo and the Quinones in either Q_A or Q_B. Tyrosine (Tyr) and the Mn_4Ca-cluster are also indicated. Figure from (Blankenship 2014), based on the 1.9Å crystal structure of PSII (Umena et al. 2011).

In PSII, the excitations are transferred to the special chlorophyll pair P680, which then donates an electron to a nearby chlorophyll (see Figure 4), creating a charge separated state; $P680^+Chl^-$. In order to prevent recombination, the distance between the charges is rapidly increased by transferring the electron to a pheophytin and then to a plastoquinone in the Q_A pocket. This semi-plastoquinone in the Q_A pocket can then donate the electron to another plastoquinone in the Q_B-pocket. The semi-plastoquinone in the Q_B-pocket waits for a second electron from the next photocycle to form plastoquinol (PQH_2), by binding two protons taken from the stromal side, and diffuses into the membrane, see also Figure 2.

The $P680^+$ is reduced in a very short time by an electron from a tyrosine of the D1 subunit, thereby forming a tyrosine radical (Styring et al. 2012), which is known as Tyr_z, see Figure 4.

The tyrosine connects P680 to the Mn_4Ca-cluster in the Oxygen Evolving Complex (OEC), where water splitting takes place. The Mn_4Ca-cluster is essential because it couples the oxidation of water, a process donating four electrons at once, with the photochemistry, which only accepts one electron each photocycle.

$$2\,H_2O \rightarrow\ O_2 + 4\,H^+ + 4\,e^-$$

The OEC accomplishes this by cycling through 5 different oxidized states, S_0 to S_4, becoming increasingly oxidized each cycle until the O_2 is released and the system returns to the first state (Kok et al. 1970).

The PQH_2 produced in the RC of PSII rapidly diffuses to a nearby Cyt b_6f complex where it binds to the Q_O site located close to the lumen, after which the two protons are released in the luminal side of the thylakoid membranes, see Figure 2. The electrons are donated to two different electron acceptors, one electron is transferred to plastocyanin (PC) via the Iron-Sulfur cluster in the Rieske subunit. The other electron travels via Cyt b_6f to the Q_i site located close to the stroma, where it reduces another plastoquinone (Q) into semiplastoquinone, which takes an electron and two protons from the stromal side and diffuses to the Q_O site, thereby releasing another two protons in the luminal side of the thylakoid membranes. This process is known as the Q-cycle and explains why each electron coming from PSII is able to facilitate the translocation of two protons from the stromal to the luminal side (Mitchell 1976).

PC is a small copper protein that facilitates the electron transfer from Cyt b_6f to the oxidized special chlorophyll pair of PSI ($P700^+$), see Figure 2. Although there is still a lot of debate, PC is most likely the long-distance electron carrier between the two photosystems since it diffuses freely in the lumen, whereas the diffusion of PQH_2 is limited to only a few PSII RCs due to molecular crowding of the photosynthetic protein complexes in the membranes (Joliot et al. 1992; Lavergne et al. 1992; Kirchhoff et al. 2000).

In PSI, like in PSII, the excitations captured by the chlorophylls are funnelled towards the RC of PSI, however the primary electron transfer most likely does not take place in the chlorophyll special pair (P700). Instead the initial electron transfer probably occurs in one of the four accessory chlorophylls (A and A_0), see Figure 5, after which the positive charge rapidly travels to P700 (Holzwarth et al. 2006; Müller et al. 2010b). This leads to a state where the P700 is oxidized ($P700^+$) and chlorophyll A_0 is reduced, after which the PC can donate an electron and reduce $P700^+$ (Hope 2000).

The chlorophyll A_0 rapidly donates the electron to one of the quinones named A_1, after which the electron is passed on to the Iron-Sulfur Clusters F_x, F_A and F_B before being transferred from PSI to the soluble protein ferredoxin (Fd) (Amunts et al. 2007). Interesting to note is that unlike in PSII where the electrons can only follow one branch (P680 -> $Pheo_{D1}$ -> Q_A -> Q_B), the electrons in PSI can follow both branches (Guergova-Kuras et al. 2001).

The Fd contains another Iron-Sulfur cluster and accepts the electron on the stromal side of the thylakoid membranes, see Figure 5. The reduced Fd travels to Ferredoxin $NADP^+$ Reductase (FNR) where the electron is donated. The FNR requires two electrons and a proton to convert $NADP^+$ into NADPH, it waits for the second electron in a system that is similar to the PSII electron transfer in the Q_B pocket. However, instead of a plastoquinone, the FNR protein contains a Flavin Adenine Dinucleotide (FAD) cofactor that performs the same function as the quinone and can be oxidized (FAD), partially reduced (FADH·) and fully reduced ($FADH_2$).

The reduction of $NADP^+$ is the final step in the electron transfer, however there is still a proton gradient ‘left’ that was produced during PSII water oxidation and proton translocation by PQH_2 and Cyt b_6f. This proton gradient, also referred to as a proton motive force, is used to produce a mechanical rotation in the ATPase-complex, the ATPase functions as a molecular motor that rotates 120° to produce one ATP, although dependent on several factors the rotation generally requires around 4 H^+ to produce 1 ATP.

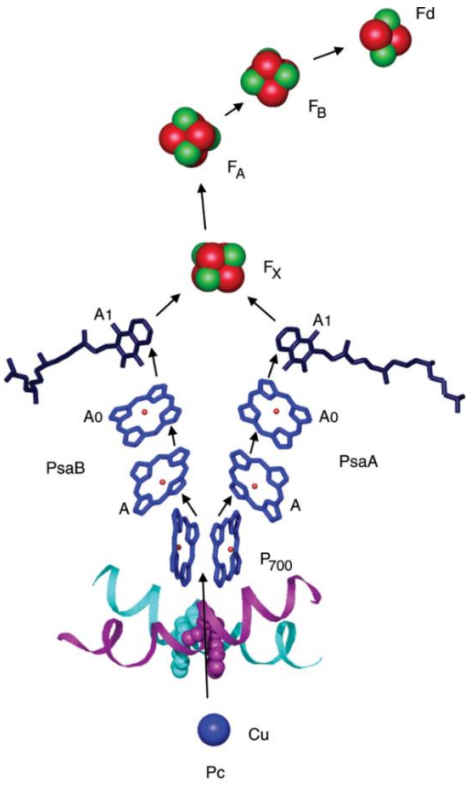

Figure 5 Model of the electron transport chain of PSI

Electrons from the cupper of PlastoCyanin (PC) in blue to the Iron-Sulfur Clusters. Figure from (Nelson and Yocum 2006).

The whole linear electron flow, starting from water and ending up in $NADP^+$, can be summarized by the so called zig-zag scheme (Z-scheme), see Figure 6. From this scheme it is clear that the two photosystems work in tandem to produce NADPH and ATP, which are then used for carbon fixation in the Calvin-Benson Cycle or end up being lost in photorespiration. The electrons coming from PSII are responsible for the creation of a proton motive force and thereby indirectly for the production of ATP, while the electrons excited by PSI lead to the production of NADPH.

Carbon fixation and photorespiration use different amounts of ATP and NADPH and since they are available in limited amounts, their productions need to be properly regulated (Sacksteder et al. 2000). If ATP is consumed faster than NADPH, there would be a shortage of $NADP^+$, which would block the linear electron flow. On the other hand, if NADPH is consumed faster than ATP, the

amount of ADP would be limiting, and ATPase would not translocate any protons. This would increase the ΔpH over the thylakoid membranes, thereby initiating Non-Photochemical Quenching, and block the proton translocation at Cyt b_6f by the oxidation of plastoquinol (Kanazawa and Kramer 2002).

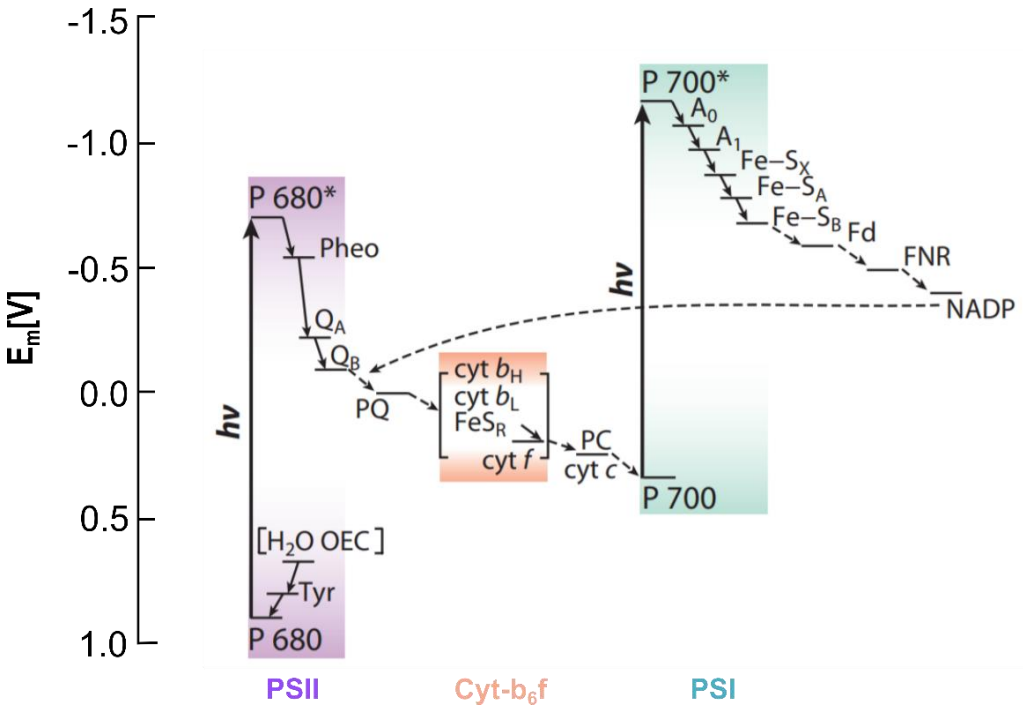

Figure 6 The Z-scheme of photosynthesis

The energetic overview of the linear electron flow in oxygenic photosynthesis named the Z-scheme, which is short for Zig-Zag scheme. Figure adapted from (Hohmann-Marriott and Blankenship 2011).

To ensure that the linear electron flow is not blocked due to a shortage of ATP or NADPH, their productions need to be finely balanced. However, under normal conditions the ratio of produced ATP/NADPH is estimated to be 1.29 (Sacksteder et al. 2000; Seelert et al. 2000), while the required ATP/NADPH ratio for CO_2 fixation, photorespiration and nitrate assimilation under normal conditions lies around 1.6 (Edwards and Walker 1983). This means that there is a structural unbalance in the production of ATP and NADPH in the linear electron flow.

Several alternative electron pathways have been documented to decrease the over-reduction of the Fd pool and keep a balance in the production of ATP and NADPH.

The most important and elusive pathway is the cyclic electron flow, a pathway where the electrons from PSI are rerouted back to the electron transfer chain, most likely somewhere upstream of Cyt b_6f (Tagawa et al. 1963; Cleland and Bendall 1992), see Figure 6. Despite the importance of the cyclic electron flow to ensure a proper linear electron flow, knowledge about specific electron transporters involved are still missing, the two main proposed pathways for the reduction of PQ involved the electron transfer from PSI to either PGR5/PGRL1 or NDH-1/NDH-2 (Yamori and Shikanai 2016). However, it is very unlikely that both PGR5/PGRL1 and the NDH-complexes are directly involved in the electron transfer. The role of NDH-1 as a direct acceptor of electrons in the cyclic electron flow is doubtful due to the low levels present with respect to PSI (Burrows 1998) and the extreme rate at which it would need to operate to keep up with the cyclic electron flow (Joliot et al. 2004; Joliot and Johnson 2011; Trouillard et al. 2012). Furthermore, no difference was observed in the qE-component of NPQ, activated by an increase in the ΔpH over the thylakoid membranes, in tobacco mutants devoid of NDH-1, suggesting that the contribution of proton translocation induced by NDH-1 is trivial (Shikanai et al. 1998). Similar observations were made for NDH-2, the algal equivalent of the NDH-1 found in plants, making it unlikely that NDH complexes are directly involved in the cyclic electron flow (Jans et al. 2008; Nawrocki et al. 2015, 2019b).

PGR5 and PGRL1, their names derived from proton gradient regulation and PGR5-like-1, were discovered to be missing in randomly mutagenized plants showing low activation of qE (Munekage et al. 2004; DalCorso et al. 2008). Their direct role as electron acceptors of PGR5 and PGRL1 are doubtful as well. PGR5 and PGRL1 have been shown to be important in the fine-tuning of the cyclic electron flow, but proved to be unnecessary for its full activation (Nandha et al. 2007; Nawrocki et al. 2017). Furthermore, both PGR5 and PGRL1 were not able to protect PSI against a sudden burst of electrons coming from PSII, making it unlikely that they are directly accepting electrons from PSI (Rantala et al. 2020).

Previously it has been shown that FNR is able to bind to Cyt b_6f and seems to perform some regulation of the cyclic electron flow (Zhang et al. 2001; Joliot and Johnson 2011; Mosebach et al. 2017). Therefore, it has been suggested that Fd itself

donates electrons to Cyt b_6f, possibly via a putative binding site located in the stroma-fluid (Nawrocki et al. 2019a). Even though the precise electron transporters of the cyclic electron flow are still under debate, it is clear that the cyclic electron flow is very important for the general function of photosynthesis, as can be seen from the stunted growth of plants lacking a well-regulated cyclic electron flow (Munekage et al. 2004; Tikkanen et al. 2010).

Light harvesting

The core complex harbouring the RC, while containing pigments for light absorption such as chlorophylls and carotenoids, are not able to capture enough light to sustain a normal growth phenotype (Polle et al. 2000; Dall'Osto et al. 2010). Therefore, the RCs are surrounded by antenna proteins, pigment-binding proteins that have a high density of chromophores per protein unit. The antenna proteins or light harvesting complexes (LHCs) not only increase the total amount of chromophores per RC, but they also have a greater variety of chromophores than the RCs, thereby increasing the absorption cross section. The excitation energies captured by the LHCs are funnelled towards the RCs where they are used to drive the electron transfer of photosynthesis. The size of the antennae systems can be easily adjusted to the specific needs of the organism, for example: in low light environments an organism will require more LHCs than an organism grown in high light. In higher plants, the LHCs are encoded by the multigenic *Lhc*-family (Jansson 1999), the LHCs connected to PSI are indicated by Lhca while those normally attached to PSII are referred to as Lhcb.

The RCs of PSII are found as dimers with three different monomeric antenna complexes, comprising Lhcb4 (CP29), Lhcb5 (CP26), Lhcb6 (CP24), directly associated with the core complex, see Figure 7. The major light harvesting antenna protein complex is LHCII, a trimeric complex connected to the core complexes via the monomeric LHCs. LHCII is the most abundant membrane protein and binds at least half of the chlorophyll (Chl) on Earth. In higher plants, three monomeric LHCs are responsible for the formation of the trimeric LHCII; Lhcb1, Lhcb2 and Lhcb3, which can form homo- or hetero-trimers. The trimerization of LHCII is responsible

for the special spectral properties of LHCII in comparison to the monomers, such as the red shifted Chl *a* (Peterman et al. 1997).

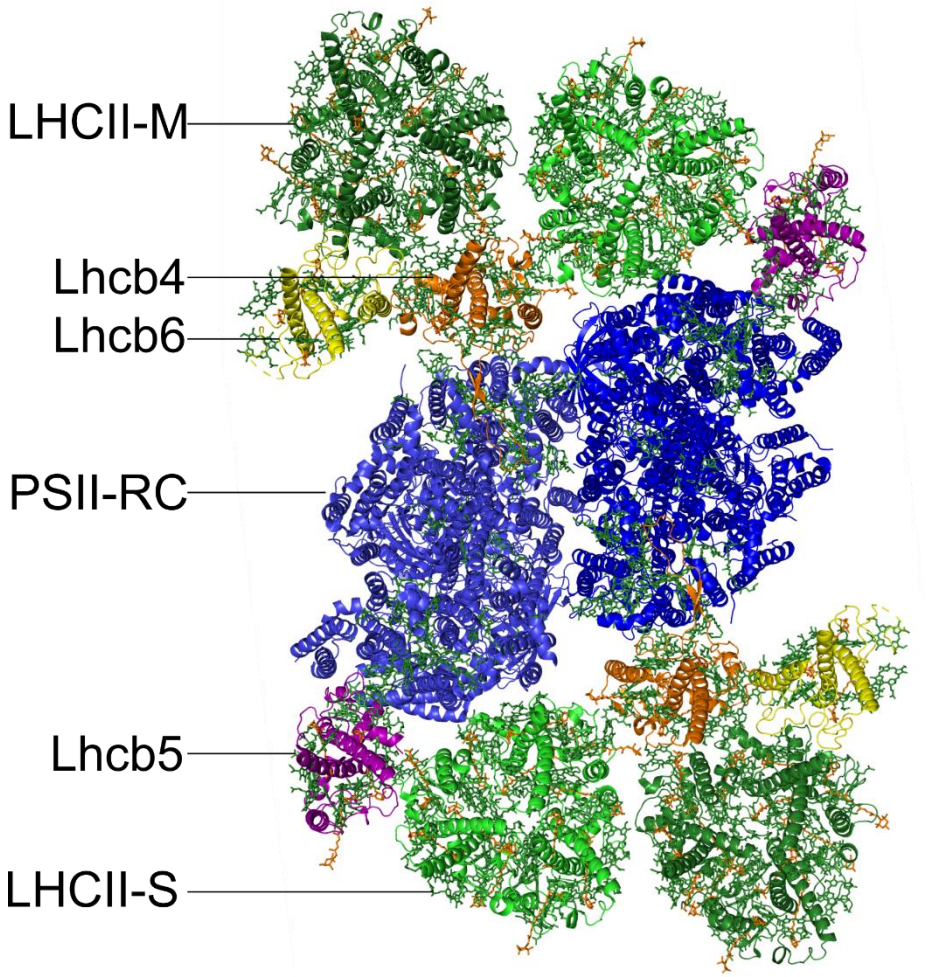

Figure 7 Overview of the structure of the dimeric PSII supercomplex

Structure of PSII-supercomplex ($C_2S_2M_2$), proteins are indicated on the left side. The structure contains two PSII-RCs indicated in light and dark blue. The minor antenna Lhcb4, Lhcb5 and Lhcb6 are depicted in orange, purple and yellow respectively. LHCII is shown in light and dark green for the strongly and moderately bound trimers (LHCII-M and LHCII-S) respectively. Structure from (Su et al. 2017).

Within the three different monomers of LHCII (Lhcb1, Lhcb2 and Lhcb3) different isoforms can be distinguished, for example *A. thaliana* contains five isoforms of Lhcb1, three for Lhcb2 and only one for Lhcb3. Each monomer of LHCII contains 8 chlorophyll a (Chl a), 6 chlorophyll b (Chl b) and 4 carotenoids; 2 luteins (Lut), 1 neoxanthin (Neo) and 1 violaxanthin (Vio) or zeaxanthin (Zea) (Liu et al. 2004).

Even though the sequences of the LHCs are very similar, they have very different roles in photoprotection and membrane organisation (Pietrzykowska et al. 2014). Lhcb1, the most abundant monomer of LHCII, is necessary for NPQ (Pietrzykowska et al. 2014). Lhcb2 on the other is essential for so called state transitions, the movement of LHCII antenna complexes to PSI RCs to balance light absorption (Leoni et al. 2013). The third monomer of LHCII, Lhcb3 is even less abundant than Lhcb2 and its deletion has so far not shown a specific difference in phenotype (Damkjær et al. 2009). While Lhcb1 and Lhcb2 can form homotrimers or heterotrimers, Lhcb3 is only found in heterotrimeric complexes. Even though LHCII is the major light harvesting protein, the monomeric proteins (Lhcb4, Lhcb5 and Lhcb6) are essential for the proper organisation of the PSII supercomplexes and highly increase the efficiency of energy transfer from LHCII to the RCs (Yakushevska et al. 2003; Caffarri et al. 2009; de Bianchi et al. 2011; Dall'Osto et al. 2019).

Interestingly land plants are the only photosynthetic organisms found to express the monomeric antenna protein Lhcb6 (CP24) (Alboresi et al. 2008), which is essential for the stable attachment of LHCII-M to the PSII supercomplex (Caffarri et al. 2009; Kouřil et al. 2013). Green algae do not have the gene for the expression of Lhcb6 and therefore have a slightly different organisation of the PSII supercomplex (Tokutsu et al. 2012; Drop et al. 2014). Interestingly gymnosperms, despite being land plants, seem to have lost Lhcb6 along the evolution and their PSII supercomplex is more similar to that of green algae (Kouřil et al. 2016). It has been proposed that an ancestor of the gymnosperms lost the gene for Lhcb6 during a long time of exposure to high light intensities (Kouřil et al. 2016). This is in line with the down-regulation of Lhcb6 found in angiosperms during high light acclimation (Ballottari et al. 2007; Kouřil et al. 2013), thereby losing the capacity to coordinate additional LHCII trimers and effectively decreasing the antenna size of PSII (Kouřil et al. 2013).

The amount of chlorophylls in the core complex of PSI, approximately 96 Chl *a* and 22 carotenoids (Jordan et al. 2001), is much higher than that found in the core complex of PSII, approximately 35 Chl *a* and 11 carotenoids (Umena et al. 2011).

The huge amount of pigments found in the core complex of PSI imply it can function efficiently without external antenna complexes. However, plants lacking LHCI, a multisubunit light harvesting complex binding to the core of PSI, show a stunted growth phenotype (Bressan et al. 2016).

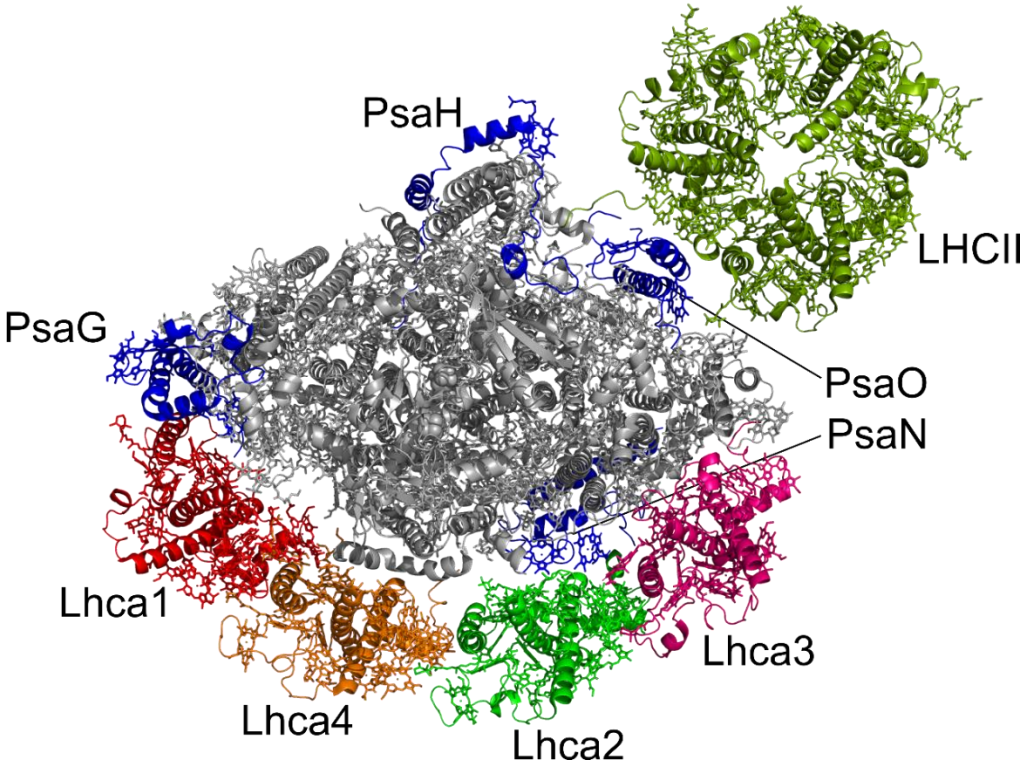

Figure 8 Crystal structure of plant PSI-LHCI-LHCII

The crystal structure of PSI with the two heterodimers Lhca1/Lhca4 and Lhca2/Lhca3 and an LHCII trimer attached on the PsaO site. Structure from (Pan et al. 2018), PDB code 5JZI.

While the core of PSI is generally found as a trimer or tetramer in cyanobacterial species (Jordan et al. 2001; Watanabe et al. 2014; Li et al. 2014), the core complex of PSI in green algae and plants is only found as a monomer (Scheller et al. 2001; Ben-Shem et al. 2003). The antenna complex supplementing PSI is generally referred to as LHCI and in higher plants comprise four monomers (Lhca1-Lhca4) that form two heterodimers (Lhca1/Lhca4 and Lhca2/ Lhca3) like a crescent shaped moon on one side of the PSI-core (Ben-Shem et al. 2003), see Figure 8. The two heterodimers of PSI are very similar to the heterodimer formed by the monomeric antenna complexes Lhcb4/Lhcb6 of PSII (Su et al. 2017). Despite the huge size of

the PSI-LHCI complex, the energy captured by LHCI is transferred to the RC in the core complex with an efficiency of almost 100% (Croce et al. 2000), thereby making PSI the most efficient photochemical energy converter (Nelson 2009; Croce and van Amerongen 2013).

In *A. thaliana* two additional *Lhca*-genes are responsible for the expression of Lhca5 and Lhca6, however these proteins are only present in large and rare PSI-NDH complexes which have been suggest to be involved in the cyclic electron flow (Peng et al. 2009).

As can be seen from Figure 8, LHCII is also able to bind to PSI, thereby increasing the total amount of chlorophylls by approximately 20% while the efficiency of energy transfer only decreases 0.2% (Wientjes et al. 2013). The interaction of LHCII with PSI is an important regulation mechanism to balance the energy absorption between the two photosystems. The spectral absorbance of the two photosystems and their antenna is quite different, where the antenna of PSI have a maximum absorption of around 682 nm, the corresponding maximum absorption of PSII is more blue-shifted (677 nm) (Caffarri et al. 2014). Besides this shift in the maximal absorbance, the PSII supercomplexes have an additional strong absorption at 650nm because of the extra Chl *b* in which LHCII is enriched. Because of this difference in the absorption spectra between PSII and PSI, changes in the spectral composition of the incoming light will directly influence the energy absorption of the photosystems and therefore the efficiency of the linear electron flow.

If PSII is overexcited there will be an overproduction of PQH_2, which cannot be completely oxidized due to the light limited turnover of PSI, that binds to the Q_0 site of Cyt b_6f which induces a conformational change that activates the STN7 kinase (Zito et al. 1999). The STN7 kinase (STT7 in green algae) phosphorylates LHCII (Bellaflore et al. 2005). LHCII in turn moves from the grana to the stroma where it attaches to PSI, thereby effectively increasing the total antenna size and the spectral range of PSI, the system is now in State II. The shuttling of LHCII is known as state transitions and can also occur the other way around, where the LHCII is dephosphorylated by TAP38/PPH1 causing it to move back to PSII (Shapiguzov et al. 2010; Pribil et al. 2010), inducing State I.

However, besides phosphorylated LHCII binding to PSI during state transitions, PSI complexes were also found to bind unphosphorylated LHCII-trimers (Wientjes et al. 2013; Bos et al. 2019). Thereby most probably simplifying the acclimation to different light intensities, because only the production of Lhcb1 and Lhcb2 would have to be regulated to increase the antenna sizes of both PSI and PSII (Ballottari et al. 2007; Wientjes et al. 2013).

NPQ and photoprotection

Plants and algae experience an extreme variability in the photon flux: at sunrise light is limiting and fully used for fuelling electron transport from H_2O to CO_2 to form sugars. At midday, however, light can be too much and the singlet Chl excited states (^{1}Chl*) produced by photon absorption cannot be quenched by photochemistry (qP) fast enough, leading to intersystem crossing (ISC) thereby producing Chl triplet states (^{3}Chl*), which react with O_2 to produce singlet oxygen ($^1O_2^*$) that can produce Reactive Oxygen Species (ROS), see Figure 9. Accumulation of ROS causes photoinhibition (qI) which drastically limits the electron transport and thereby plant growth.

In order to protect themselves, plants and algae have multiple protection mechanisms to decrease or prevent the damaging effects of excess light, these include minimising light absorption, mitigation of ROS and dissipation of excess energy. The fastest response, seconds to minutes, is called energy quenching (qE) and is a Non-Photochemical Quenching (NPQ) process that dissipates the excess energy as heat. Activation of qE is directly controlled by the pH of the thylakoid lumen, which become acidic under excess light due to an increased electron transfer from PSII to PSI, thereby pumping protons to the lumen.

In higher plants and green algae, two proteins are essential for sensing the lower pH and activation of qE; LHCSR and PSBS, found in algae and plants respectively. The main difference between these proteins is that LHCSR contains pigments while PSBS does not (Richard et al. 2000; Dominici et al. 2002; Pinnola et al. 2015). The precise mechanism of how the energy is quenched is still the object of a lively debate, however a common ground for all theories are pigment-pigment interactions

between chlorophylls and xanthophylls (Holt et al. 2005; Ahn et al. 2008; Müller et al. 2010a). This means that LHCSR is most likely not only involved in the activation of qE but also the site of quenching, while PSBS, due to a lack of pigments (Dominici et al. 2002), must interact with antenna proteins, most probably Lhcb4 and LHCII (Dall'Osto et al. 2017).

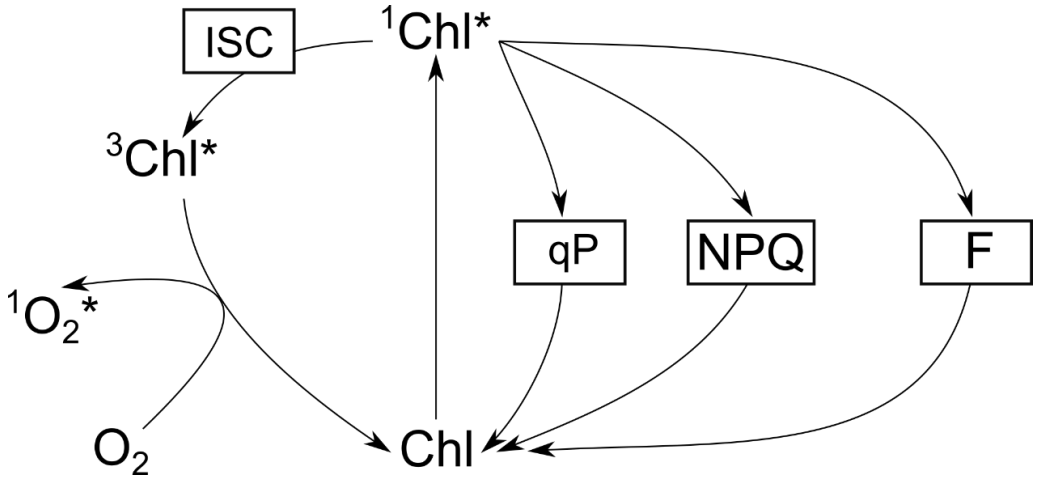

Figure 9 Schematic overview of the chlorophyll dissipation pathways

After excitation of a chlorophyll, it can follow multiple pathways back to the ground state where it can be re-excited. photochemistry (qP), formation of chlorophyll triplets via Inter System Crossing (ISC) leading to the production of ROS, dissipation as heat by Non-Photochemical Quenching (NPQ) or by re-emitting a photon as fluorescence (F). The fluorescence can be easily measured and is used to distinguish the different components of NPQ.

Besides the activation of either PSBS or LHCSR, the low pH also activates Violaxanthin De-Epoxidase (VDE), which de-epoxidates violaxanthin into antheraxanthin and subsequently into zeaxanthin (Demmig-Adams and Adams 1996). Zeaxanthin induces photoprotection by scavenging ROS due to the strong antioxidant properties (Krinsky 1979; Edge et al. 1997; Havaux et al. 2007) and by enhancing qE (Niyogi et al. 1998). The induction of the zeaxanthin dependent quenching (qZ) is slower than qE and can take minutes to tens of minutes, furthermore it is species-dependent, where plants and mosses rely on the formation of zeaxanthin (Dall'Osto et al. 2005; Pinnola et al. 2013), the green alga *C. reinhardtii* does not (Bonente et al. 2011; Dinc et al. 2016). Deactivation of qZ

occurs via the reconversion of zeaxanthin into violaxanthin by the enzyme zeaxanthin epoxidase (ZEP), which can take several tens of minutes or even hours, depending on the conditions.

Another process that can decrease the chlorophyll fluorescence are state transitions (qT). Since chlorophyll fluorescence is normally measured at room temperature where the contribution of PSI fluorescence is minimal, the fluorescence measured largely comes from PSII and its antennae proteins. Therefore, if part of the antennae proteins move from PSII to PSI, the fluorescence will decrease and part of the captured energy will be transferred to PSI (Nawrocki et al. 2016). However, qT in general, only has a small contribution to the overall quenching and is mainly present in low light (Maxwell and Johnson 2000).

Although technically not an NPQ process, decreasing the total amount of light absorbance is an effective way of reducing the damaging effects of oversaturation. In plants the movement of chloroplasts (qM) is induced by white or blue light by the phototropin receptor (Phot2) (Kasahara et al. 2002). The chloroplast movement entails that all the chloroplast move to the side, thereby shading each other and effectively decreasing the total amount of light absorbed, which leads to a reduction in fluorescence (Cazzaniga et al. 2013). The chloroplast movement can take up to 30min to be fully activated and has been shown to be an important additional photoprotection mechanism for plant survival (Kasahara et al. 2002; Cazzaniga et al. 2013). The chloroplast relocation system of plants is similar to the phototactic behaviour of green algae which can ‘swim’ towards or away from a light source (Wakabayashi et al. 2011).

Recently another form of sustained quenching has been identified (Malnoë et al. 2018), the quenching component is termed qH and is especially slow in its relaxation, a 10min induction takes 3 hours to relax. The precise mechanism of action is still unknown, but it is induced by the plastid lipocalin LCNP, whereas the suppressor of qH is SOQ1 (Brooks et al. 2013; Malnoë et al. 2018). LCNP expression is up-regulated during abiotic stresses such as high light and drought (Levesque-Tremblay et al. 2009). The current hypothesis about qH states that there

is a conformational change in the PSII antenna proteins from a light-harvesting into a quenched state (Brooks et al. 2013; Malnoë et al. 2018).

The last component is termed qI, for photoinhibition and technically describes the inactivation of PSII caused by excess light and usually takes days before it relaxes, e.g. before all damaged proteins and chromophores have been repaired or replaced. Slower responses induced by zeaxanthin formation (qZ), chloroplast movement (qM), sustained quenching (qH) and photoinhibition of the RC (qI) are generally collectively designated as qI due to the difficulty of distinguishing these components in general chlorophyll fluorescence measurements without the use of inhibitors such as DTT and chloramphenicol

The Calvin-Benson Cycle

The NADPH and ATP produced in the linear electron flow of the oxygenic photosynthesis are used in the Calvin-Benson Cycle to fix carbon, coming from CO_2, into carbohydrates. The carbohydrates can either be used, stored for later usage or transported via the vascular system.

The Calvin-Benson cycle starts with the carboxylation of CO_2 and Ribulose-1,5-Bisphosphate (RuBP) into 3-phosphoglycerate (3PGA) by the protein Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase also known as RuBisCO, see Figure 10. RuBisCo is the most abundant protein on earth and comprises roughly 20% of the total amount of proteins in plants. After the carboxylation step, the 3PGA is converted into glyceraldehyde-3-phosphate (G3P) that can be converted into glucose via the gluconeogenesis pathway. The remaining G3P not used for the gluconeogenesis is reconverted into ribulose-1,5-bisphosphate to start the next cycle of the carboxylation.

Even though RuBisCO is responsible for almost all the carbon fixation on earth, the turnover rate is extremely slow, around 1 to 10/s (von Caemmerer and Quick 2000). The slow turnover rate (k_{cat}) explains the huge abundance of RuBisCO found in plants.

However, not only the slow turnover rate, but also the fact that RuBisCO can bind O_2 as a substrate greatly reduces the efficiency of RuBisCO. When RuBisCO binds O_2 instead of CO_2, the O_2 reacts with RuBP (5 carbons) and 2-P-Glycolate (2 carbons) is produced along with 3PGA (3 carbons), see Figure 10.

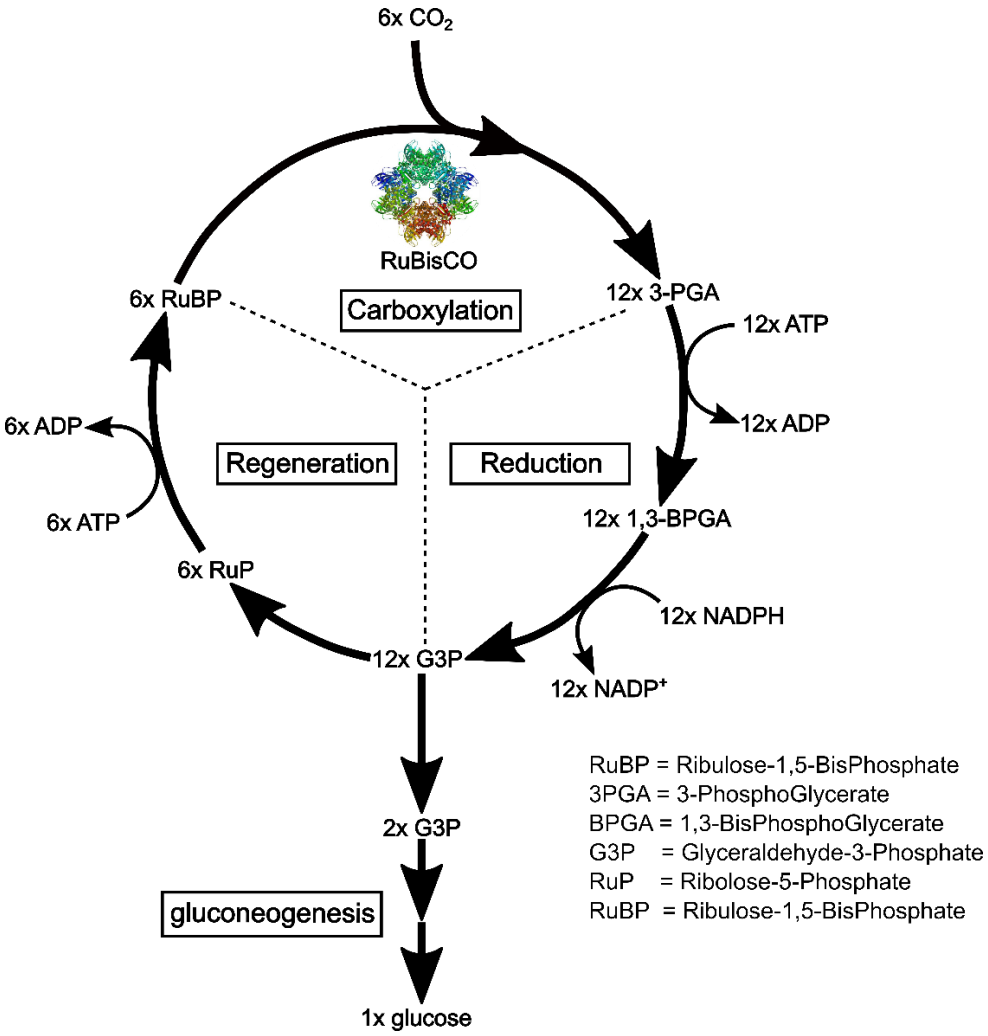

Figure 10 Overview of the Calvin-Benson cycle

Overview of the Calvin-Benson cycle. Structure of RuBisCO from (Taylor and Andersson 1997), pdb-code 1AUS.

The 2-P-Glycolate can be recycled via the respiratory pathway, sometimes also referred to as the C2 photosynthesis, which most likely evolved alongside the oxygenic photosynthesis in the cyanobacteria (Canfield 2005; Allen and Martin 2007; Eisenhut et al. 2008). The 2-P-Glycolate and the glycolate, to which the 2-P-Glycolate is converted before being recycled into 3PGA, are toxic (Anderson 1971)

and the recovery of the 2-P-Glycolate into 3PGA requires energy in the form of ATP and leads to the loss of previously assimilated CO_2. The glycolate is transported to the peroxisome where it is converted into glyoxylate with the release of hydrogen peroxide, another toxic compound.

The glyoxylate can be converted into glycine via two different pathways, either using glutamate or serine after which the glycine is transported into the mitochondria. Two glycines are used to produce one serine, which leads to the loss of CO_2 and NH_3. The serine is transported back to the peroxisome where it is converted into glycerate, which is then transported into the chloroplast and reconverted into 3PGA requiring one ATP. In the end no net NADH is used for the recovery of the 2PGA, however, every 2 oxygenation events require the regeneration of 2 RuBP, leading to an overall cost of 5 ATP and 3 NADPH for every 2 oxygenation events.

Even though RuBisCO has a much higher affinity for CO_2, the concentrations of CO_2 and O_2 in the atmosphere (approximately 0.04% and 21% respectively) and cellular tissue still lead to many photorespiration processes (the reaction of RuBP with O_2) producing high quantities of 2PG during the day (Bauwe et al. 2010). Approximately one photorespiration event occurs for every 5.7 carboxylation reactions, leading to a carbon loss of 21% from the net CO_2 assimilation (Cegelski and Schaefer 2006). The ratio of carboxylation/oxygenation gets even worse in higher temperatures due to the decreased affinity of RuBisCO for CO_2 (Jordan and Ogren 1984). Besides the decreased affinity, the stomata tend to close in warm conditions to reduce the loss of water, leading to a decreased level of CO_2 inside the cells and therefore more photorespiration.

The efficiency of RuBisCO has never been improved over the millions of years of evolution, indicating that RuBisCO is a protein with a unique and indispensable function for carbon fixation. However, during evolution an alternative has evolved to decrease the rate of photorespiration. This alternative is based on concentrating the CO_2 in the vicinity of RuBisCO and thereby circumventing photorespiration.

This alternative is known as the C4 photosynthesis, referring to the length of the primary carbon-chain upon the first carboxylation of CO_2. As can be seen Figure 10,

every RuBP reacts with a CO_2 to produce two 3PGA, which is a 3-carbon chain, hence the name C3 photosynthesis. However, the C4 plants have targeted the Calvin-Benson cycle to the bundle sheath cells, that have been cut off from the external air by the mesophyll cells surrounding them. The mesophyll cells first carboxylate HCO_3^- with phosphoenolpyruvate (PEP) into oxaloacetate using the enzyme PEP-carboxylase, see Figure 11. The oxaloacetate is a 4-carbon chain, hence the name C4 photosynthesis. Since PEP-carboxylase uses HCO_3^- as a substrate and not CO_2 or O_2, it is unable to perform oxygenation.

HCO_3^- is in equilibrium with H_2O and CO_2, but under normal circumstances it takes too long to reach this equilibrium for an efficient PEP-carboxylase activity.

$$H_2O + CO_2 \rightleftharpoons HCO_3^- + H^+$$

To increase the speed with which the equilibrium is reached, the reaction is catalysed by the enzyme carbonic anhydrase, one of the fastest enzymes known, of which the rate is mostly limited by the rate of diffusion (Lindskog 1997).

After the formation of oxaloacetate, it is converted into malate and transported via the plasmodesmata to the bundle sheath cells where it releases CO_2 to produce pyruvate. This pathway can concentrate the CO_2 more than 15 times the atmospheric concentration, virtually eliminating photorespiration. Although recycling of pyruvate into PEP requires 2 ATP, the avoidance of photorespiration still overcomes the cost of the 2 ATP in many different conditions (Hatch 1987). Another interesting addition to the reduction of photorespiration found in most C4 plants such as maize and sugarcane is the absence of PSII in the bundle sheath cells. Meaning that there is no oxygenic photosynthesis, but only the cyclic electron flow and thus no production of O_2. However, this leads to an imbalance of NADPH production, which is circumvented by a transport of 3-PGA to the mesophyll cells where it is reduced to G3P before being transported back into the bundle-sheath cells (Hatch 1987).

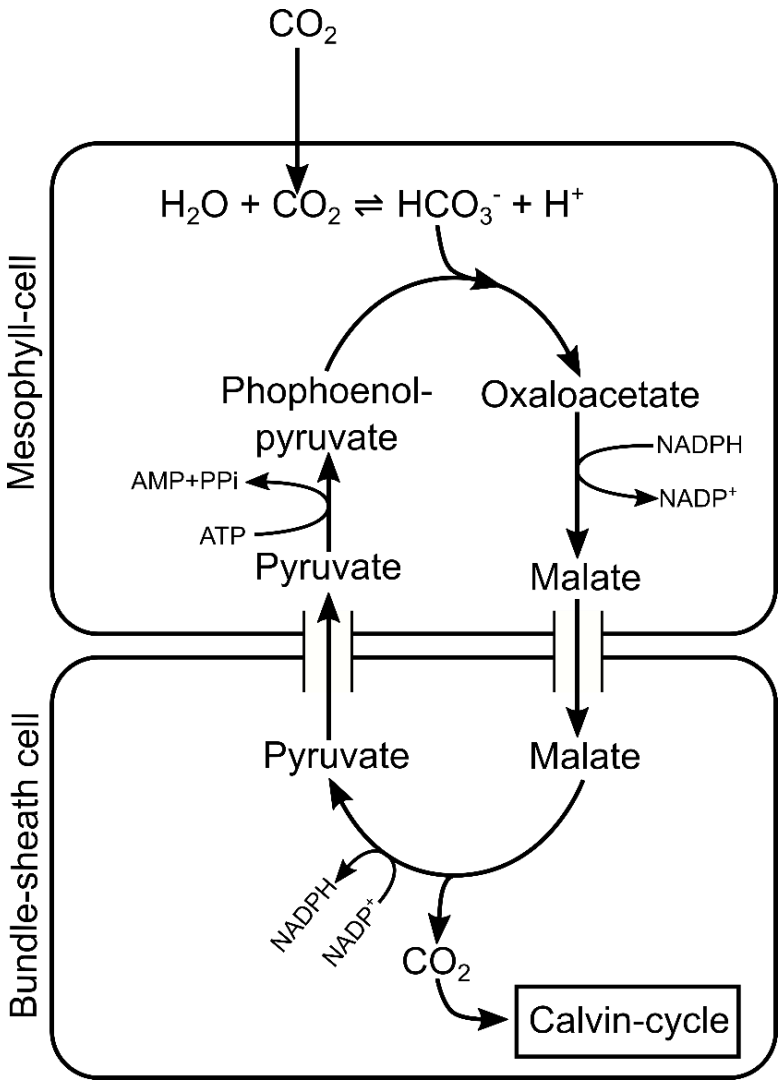

Figure 11 Pathway of the C4 photosynthesis

Schematic representation of the C4 photosynthesis pathway. Figure adapted from (Blankenship 2014)

A second alternative is known as the Crassulacean Acid Metabolism (CAM), so named due to the discovery of it found in the *Crassulaceae* family. CAM-photosynthesis is mainly found in plants living in arid environments, such as cacti. The system they use is similar to the C4-photosynthesis, however, these plants don't have the separation of mesophyll and bundle-sheath cells like C4 plants do but separate the two processes in time. During the night, when temperatures are cooler, the stomata are opened, and CO_2 is stored in the form of malic acid in the vacuoles.

During the day the stomata are closed, and the malic acid is used to release the CO_2 for the Calvin cycle. Interestingly, the CAM-photosynthesis is also found in some aquatic plants. Here the function is not related to limiting the loss of water, but serves to harvest CO_2 during the night, when there is no competition for CO_2 harvesting by algae and cyanobacteria (Keeley 1998).

In contrast to plants, most algae and cyanobacteria use active pumping mechanisms to concentrate CO_2. To ensure that CO_2 doesn't leak back, it is stored in the form of HCO_3^- which cannot penetrate the membrane due to the negative charge. In cyanobacteria and algae, the RuBisCO is densely packed, together with carbonic anhydrase, in specific compartments called carboxysomes or pyrenoid bodies respectively. The carbonic anhydrase reconverts the HCO_3^- into CO_2 after which it is immediately used by the densely packed RuBisCO (Wang et al. 2011; Kupriyanova et al. 2013).

Increasing crop productivity by optimizing photosynthesis

The global crop productivity has increased by 135% between 1961 and 2005, this increase has been driven by intensive breeding programmes, usage of pesticides, fertilizers and by mechanization and irrigation (Burney et al. 2010; Pingali 2012). However, in recent years the increase in crop yields are stagnating.

A major possibility to increase crop productivity is to directly target photosynthesis processes since the theoretical maximal photosynthetic energy conversion efficiency in the most optimal conditions is only 4.6% or 6.0%, in C3 and C4 plants respectively (Zhu et al. 2010), see Figure 12. The actual biomass production based on the incident solar radiation is much lower, with highest reported values being 2.4% and 3.7% for C3 and C4 plants respectively during a full growing season (Monteith 1977; Beadle and Long 1985; Piedade et al. 1991; Beale and Long 1995).

Photosynthetic organisms can only absorb a small portion of the incoming sunlight, the Photosynthetically Active Region (PAR) which ranges from 400nm to 700nm, the rest (51.3%) falls outside the photosynthetically active spectrum (Zhu et al. 2008), see Figure 12. Besides the light outside of the PAR, green light is hardly

used for photosynthesis due to the properties of chlorophylls that only absorb strongly in the blue and red regions of the spectrum, leading to a loss of 4.9% of the total incoming solar light (Zhu et al. 2008). Another loss has been termed photochemical inefficiency, even though blue light is strongly absorbed and has 75% more energy than the red photons, the blue excited states of chlorophylls are very short-lived (ps) and fall back in femtoseconds to the lower excited state of the red photons, accompanied by the release of heat, leading to an energy loss of 6.6% (Zhu et al. 2008).

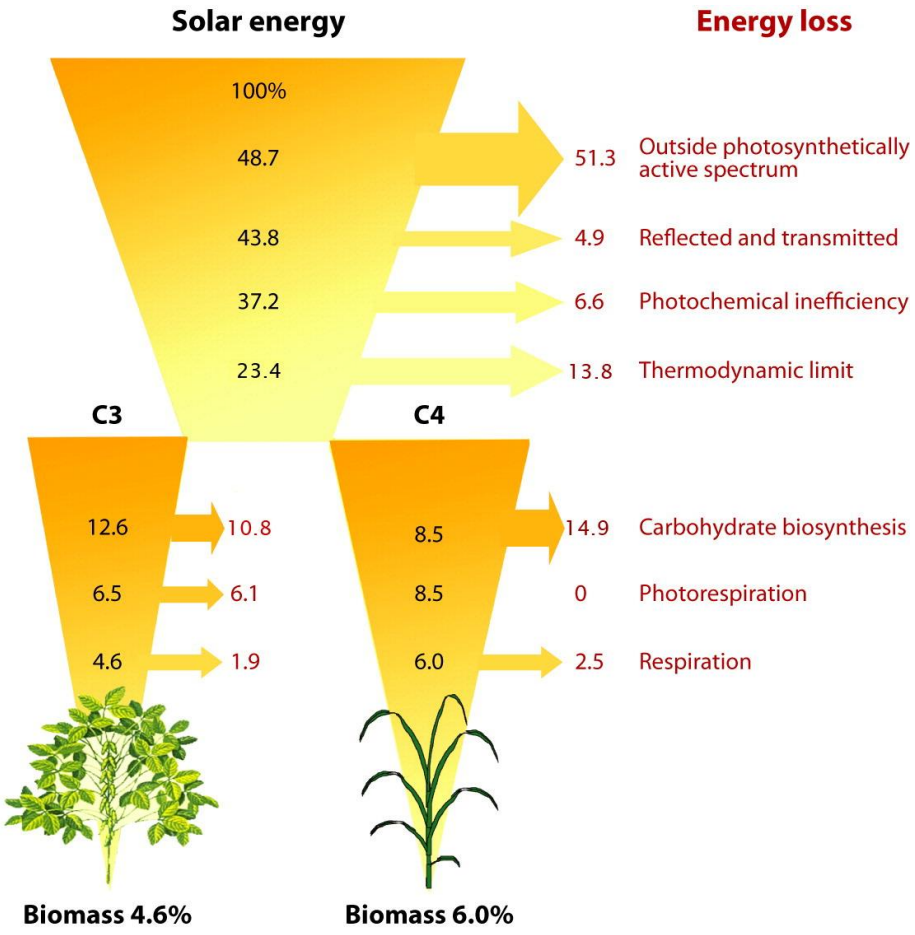

Figure 12 The maximal photosynthetic energy conversion efficiency

The losses at each step are given in red. For the CO_2-fixation the path is split in two for either C3 or C4 plants. Figure is from (Zhu et al. 2010).

Some algae species, such as the *Eustigmatophytes*, are able to shift the absorbance of chlorophyll *a* by modifying the protein environment of the chromophores (Wolf et al. 2018). Even plants are able to absorb beyond 700nm and drive the oxygenic photosynthesis (Pettai et al. 2005), however the absorbance is low and plants only subjected to far red light eventually die. Especially the antennae of PSI contain some red-shifted chlorophyll *a* (Wientjes et al. 2012) that can absorb wavelengths of up to 738nm (Croce et al. 1996). Since a large part of sunlight consists of far-red light in the region of 700-750nm, expanding and increasing the absorption cross section of plants might be an interesting opportunity to increase crop yield. Especially since the light beneath a canopy is highly enriched in the far-red spectrum (Kasperbauer 1987), which could be useful for the lower leaves in densely cultivated crops. Ideally if all the photons from sunlight in the region of 700-750nm could be used for photosynthesis, this would lead to a 19% increase in the total amount of absorbable photons (Chen and Blankenship 2011). Even though the photons in the far-red light have less energy, as long as the energy is enough to support photochemistry, this should not be a problem and since many cyanobacteria are already able to perform photosynthesis in far-red light, it should be possible to engineer in plants as well. Creating plants that are able to produce Chlorophyll d and/or f would cause the absorption cross section to shift towards the red, however this would require the introduction of antenna complexes from prokaryotes into eukaryotes, which is not an easy or maybe even an impossible task. Another possibility is to modify the already present antenna proteins in plants to mimic the far-red shift that has been observed in *Eustigmatophytes* (Wolf et al. 2018). So even though some photosynthetic organisms show that it is possible to expand the photosynthetically active region towards the far-red light and that this would theoretically greatly increase the total amount of incoming light, approximately 19% (Chen and Blankenship 2011), this still requires a much deeper understanding of the processes involved in light-harvesting and photochemistry.

Another possibility is to target the CO_2 fixation itself, C4 crops are in general much more efficient than C3 crops, see Figure 12. Since only 3% of the plant species use the C4 photosynthesis and a lot of the crops which are cultivated are C3 plants, great crop improvements could be made in targeting the CO_2 fixation.

Several efforts are being made to introduce the C4 photosynthesis CO_2 pumping mechanisms in C3 crops (Schuler et al. 2016). Other efforts are more focused on elucidating, and introducing the CO_2 concentrating mechanisms from green algae into plants (Mukherjee et al. 2019).

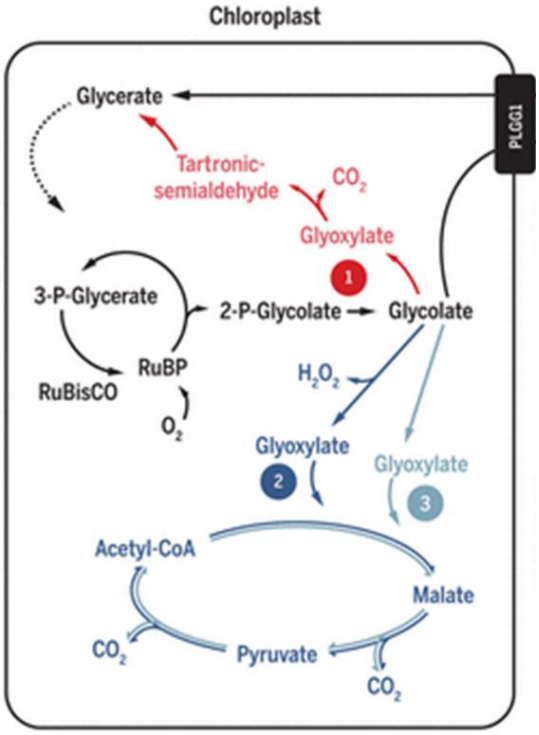

Figure 13 Alternative photorespiration pathways

Alternative photorespiration pathways, figure from (South et al. 2019).

However, these targets aim to decrease the probability of photorespiration, an alternative would be to modify the classic photorespiratory pathway by introducing specific sets of genes from plants or bacteria, targeted toward the chloroplast, to decrease the cost of photorespiration (Kebeish et al. 2007; Maier et al. 2012; South et al. 2019). Recently three alternative pathways were compared to each other in the C3 model crop organism: *Nicotiana tabacum*.

The first pathway is an alternative glycolate pathway from *E. coli*, which consists of 5 genes in total; glycolate dehydrogenase, glyoxylate carboligase and a tartronic semialdehyde reductase (Kebeish et al. 2007). The *E. coli* glycolate dehydrogenase, consisting of 3 subunits, does not produce hydrogen peroxide like the glycolate

oxygenase of plants in the peroxisome. Then two glyoxylates are combined into one tartronic semi-aldehyde which is accompanied by the release of CO_2, and the tartronic semi-aldehyde is converted into glycerate which can be used in the Calvin-Benson cycle. A similar pathway, to the first pathway introduced, has been found in the cyanobacterium *Synechocystis* (Eisenhut et al. 2006).

The second alternative pathway is targeting the glycolate oxidase, found in the peroxisomes of *A. thaliana*, to the chloroplasts (Maier et al. 2012). This was done in combination with an *E. coli* catalase to avoid the hydrogen peroxide accumulation that can have negative effects on growth (Fahnenstich et al. 2008). To convert glyoxylate, a malate synthase gene from *Cucurbita maxima*, was targeted toward the chloroplasts to convert glyoxylate together with acetyl-CoA into malate. The malate is decarboxylated into pyruvate, releasing the first CO_2 and then the pyruvate is converted into acetyl-CoA with the release of another CO_2, this is all done by genes already present in the chloroplast (NADP-malic enzyme and pyruvate dehydrogenase). Introduction of these three genes increased the fresh weight of *A. thaliana* by 28%, caused by an increased leaf production. The advantages of introducing this pathway were almost completely negated in CO_2 levels of 2000ppm (around 5 times more than normal), indicating that indeed the expression of these genes is helping to reduce photorespiration in *A. thaliana*. However, when this pathway was introduced into *N. tabacum*, the advantage was minimal and in many times resulted in phenotypes with a stunted growth (South et al. 2019).

The third pathway relies on the replacement of the glycolate oxidase from the pathway 2 by a glycolate dehydrogenase from *C. reinhardtii* (Aboelmy and Peterhansel 2014), omitting the need for the *E. coli* catalase. Alone this pathway was already able to increase the biomass of *N. tabacum* by 25%, however by increasing the flux of glycolate through the synthetic pathway by blocking one of the glycolate exporters (PLGG1), increased this even further to 41% (South et al. 2019).

The theoretical biomass production of 4.6% and 6.0% is never achieved, not even close, which is mainly due to the limited capacity of photosynthetic organisms to

use all the sunlight that is received by the leaf. Photosynthesis only increases linearly until about a quarter of the sunlight, above photosynthesis will move to a plateau which is reached at about half of the total amount of sunlight. Above about one quarter of the sunlight, part of the energy will be quenched and dissipated as heat. The need for this type of regulation can be explained based on the extreme variability of photon flux received by the leaves: at sunrise light is limiting and fully used for fuelling electron transport (ET) from H_2O to CO_2 to form sugars. At midday, however, light can be too much and the ^{1}Chl* excited states produced by photon absorption cannot be quenched by photochemistry fast enough, leading to intersystem crossing to the ^{3}Chl*, which reacts with O_2 and results in the synthesis of Reactive Oxygen Species (ROS). ROS accumulation causes photoinhibition of photosynthesis which drastically limits plant growth and therefore must be avoided. Dissipating light when in excess and using even the last photon under low light is a difficult exercise which is essential to ensure the maximal growth rate. However, plants are more "interested" in surviving stress and reproducing than in growing big and since most of the stresses include a photo-inhibitory component negative effects such as cold, heat and drought on crop yield is in large part mediated by photoinhibition and account for productivity losses (Nishiyama and Murata 2014). Thus, plants have developed a hysteretic response to light: in order to avoid damage, plants over-regulate energy dissipation thus growing less than could be afforded under farming conditions where the abiotic stresses are limited. This indicates that there is large room for engineering energy dissipation and increase crop production, as shown by seminal work (Kromdijk et al. 2016).

Since the light is extremely variable over the day due to canopy movement or clouds passing before the sun, plants constantly have to change between an energy dissipating state in full-sunlight and an energy harvesting state when the light intensity drops. There are several time scales for photoprotection mechanisms, generally referred to as NPQ-mechanisms. The fastest is energy quenching (qE) that is activated by PSBS and greatly enhanced by the conversion of violaxanthin into zeaxanthin (qZ). However, activation and especially the de-activation takes time and can lead to significant losses in the total amount of energy captured, with losses of CO_2 fixation estimated between 7.5% and 30% (Long et al. 1994; Werner

et al. 2001; Zhu et al. 2004). In a recent work three proteins involved in NPQ were overexpressed, PSBS responsible for qE activation and violaxanthin de-epoxidase (VDE) and zeaxanthin epoxidase (ZEP) which are involved in the xanthophyll cycle (qZ). By overexpressing these proteins, both the activation and especially the relaxation of NPQ have been significantly increased which has led to an increased biomass production of 15% in field grown tobacco plants (Kromdijk et al. 2016). However, upregulation of the same proteins (PSBS, VDE and ZEP) in *A. thaliana* led to a decrease in biomass accumulation even though the activation and relaxation was faster in the transformed lines (Garcia-Molina and Leister 2020). Therefore, improvements in photosynthesis can have great variances in different species making the task even more complicated.

As can be seen from the above described works, several strategies have already been employed to (greatly) improve the biomass production of crops by modifying photosynthesis, making photosynthesis an interesting target to deal with the increasing demand for food production. However, much more research will be necessary in the fundamental elucidation of photosynthesis and energy transfer in order to effectively increase the crop productivity.

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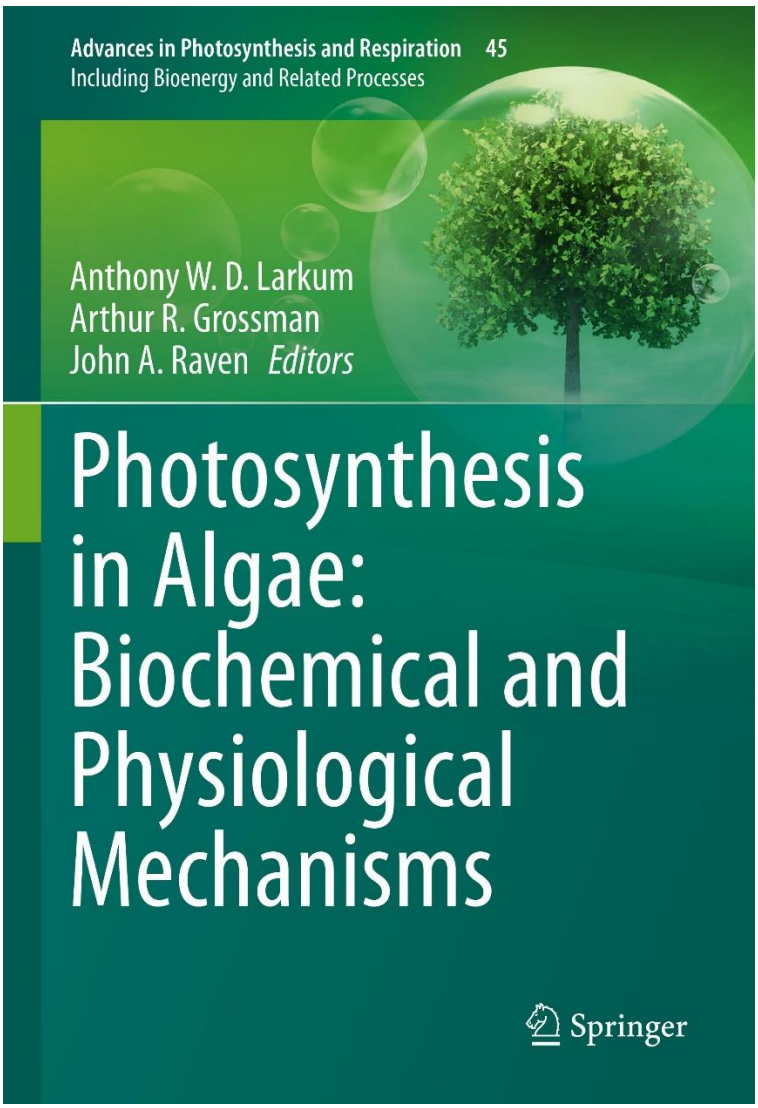
Chapter 1

Chlorophyll-Xanthophyll Antenna Complexes: In Between Light Harvesting and Energy Dissipation



A version of this chapter was published in:

Schiphorst, C., & Bassi, R. (2020). Chlorophyll-Xanthophyll Antenna Complexes: In Between Light Harvesting and Energy Dissipation. In A. W. D. Larkum, A. R. Grossman, & J. A. Raven (Eds.), *Photosynthesis in Algae: Biochemical and Physiological Mechanisms* (pp. 27–55). Springer International Publishing. https://doi.org/10.1007/978-3-030-33397-3_3

Chlorophyll-Xanthophyll Antenna Complexes: in between Light Harvesting and Energy Dissipation

Christo Schiphorst and Roberto Bassi

Department of Biotechnology, University of Verona, 37134 Verona, Italy

Abstract

Photosynthetic organisms face a constant dilemma: harvesting as much light as possible while minimizing damage when in excess light conditions. Environmental conditions reproducibly change during the day while sudden light changes superimpose due to shading and require quick responses to ensure maximal growth. Pigment-binding Antenna proteins fulfil both roles by ensuring efficient light harvesting, funnelling excitation energy towards the reaction centres while dissipating excess energy in the form of heat when necessary and scavenging high energy species as a further defence. Oxygenic organisms exhibit a highly diversified complement of antenna proteins in striking contrast with the photosynthetic reaction centres, which are highly conserved. This can be attributed to the adaptation to different light regimes found on Earth. Of particular importance is the mechanism called Non-Photochemical Quenching (NPQ) catalysing energy dissipation by regulating the interactions between chlorophylls and carotenoids, likely by multiple reactions involving excites states. During the evolution different proteins have fulfilled this function including LHCSR, found in most algae, until PSBS evolved in advanced green algae.

Introduction

In oxygenic photosynthesis light is captured and used to drive electrons from H_2O to NADPH, meanwhile building a proton gradient through the thylakoid membrane for ATP synthesis, and in the process water is split and oxygen is liberated to the environment. Photons are absorbed by proteins binding chlorophylls (Chls) and carotenoids (Cars) which, besides absorbing photons, also catalyse redox reactions

to feed electron transport and a variety of photoprotective processes for coping with the highly variable nature of solar radiation.

Photosynthetic reaction centres (RCs) come in two sorts, depending on the type of electron acceptors: a quinone, in type II, and an Iron-Sulphur cluster in type I (Nitschke and William Rutherford, 1991). These two RC-types, also referred to as Photosystem II (PSII) and Photosystem I (PSI), work in tandem and lead to oxygen production and linear electron transport. In each RC the excitation of a chlorophyll pair, either P680 or P700, leads to an oxidized special pair which is then neutralized by an electron from, respectively H_2O or plastocyanin. The electrons from the primary reductants enter the linear electron transport chain to reduce $NADP^+$.

The pigments for light harvesting and chromophores for electron transport are bound to transmembrane protein complexes called "Core Complexes", including the special pairs, which together form the RCs. Even though the RCs of the two photosystems are photosynthetically competent, as shown in core complex-only mutants, e.g. *ch1* in plants and *Cao* in algae (Dall'Osto *et al.*, 2010; Polle *et al.*, 2000) such plants show effective, yet reduced growth, because the light harvesting capacity is low, limiting the period of efficient electron transport rate to midday. In order to widen the photosynthetic efficient window, the RCs are endowed with antenna proteins: pigment-proteins which have a far higher density of chromophore per protein scaffold unit. Antenna proteins enhance the absorption cross section in two ways: i) they harbour a greater variety of chromophores absorbing over different spectral intervals; and ii) they increase the overall number of chromophores feeding the captured energy to each RC. Besides obvious advantages, antenna systems have backsides: building of pigment-protein arrays is energetically expensive while the daily variations in photon flux, not mentioning canopy movement, clouds passing before the sun, or waves in the sea, cannot be matched by a parallel dismantling and re-synthesis of antenna proteins. In addition, the subsequent reactions of linear electron transport chain occur in a wide kinetic range. Since plastoquinone is reduced to plastoquinol by PSII in ns, its oxidation by Cyt b_6f occurs in ms, excess light therefore over-reduces plastoquinone leading to charge recombination, chlorophyll triplet state (^{3}Chl*) accumulation and reaction

with O_2, causing reactive oxygen species (ROS), especially in PSII, singlet oxygen ($^1O_2$*) production and photoinhibition. Similarly, superoxide is readily produced when the Calvin-Benson cycle is saturated and the flux of reductants from PSI exceeds the capacity of downstream metabolic reactions. Indeed, the high energy univalent reducing energy transfer (ET) intermediates obviously react with oxygen whenever they accumulate for lack of oxidized electron partners, creating ROS species, principally H_2O_2 and leading to photodamage. In this chapter we will review examples of how oxygenic photosynthetic organisms evolved a variety of different regulative, acclimative and ROS-scavenging mechanisms which are activated depending on light conditions. These prevent and/or reduce the damaging effects of excess light and the consequent oxidative stress while optimizing light harvesting.

Chromophores

Light harvesting is based on chromophores, i.e. pigments attached to proteins. These come in many different types depending on species. The key chromophore and most abundant in oxygenic photosynthesis is chlorophyll (Chl).

Chlorophylls absorb strongly in the blue (350-500nm) and red regions (625-700nm) of the visible light spectrum. The absorption peaks in the blue are named Soret-bands, or B-bands, while peaks in the red region are named Q-bands, see Fig. 1. Small differences in chromophore structure can have a drastic effect on the absorption spectrum (Chen and Blankenship, 2011; Blankenship, 2014). For example, the difference between Chl *a* and Chl *b*, consists of an aldehydic substituent of the tetrapyrrole ring structure *vs* a methyl group. This causes a strong red shift of the Soret bands and a blue shift of the Q bands, see Fig. 1. Besides the chemical structure, the environment greatly influences the Chl absorption spectrum by shifting or broadening the bands. This is an essential feature used by organisms to widen the spectral range of light harvesting towards wavelengths available in specific environmental niches (van Amerongen *et al.*, 2000; Wientjes *et al.*, 2012). Small changes in the binding of chromophore to a protein, i.e. the conformation of the pigment-protein, can drastically change the absorption and emission spectra of

the chromophores, as shown for example by a up to 35nm shift in the emission spectrum in the LHCA4 protein, an antenna protein bound to PSI in plants and respective orthologues in algae (Morosinotto *et al.*, 2003; Wientjes *et al.*, 2012; Bassi *et al.*, 1992). Moreover, coordination in the protein scaffolds ensures optimal orientation and distance for efficient energy transfer.

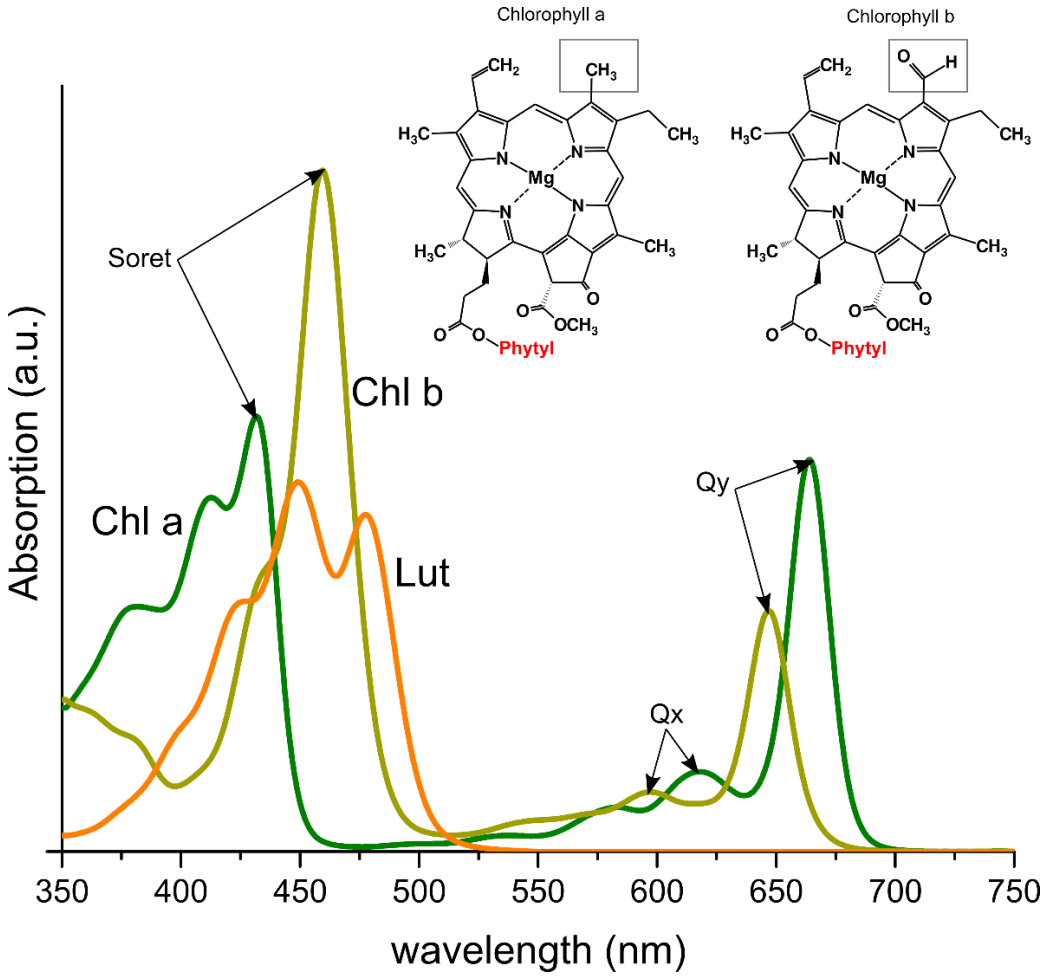


Figure 1. *Absorption spectra of Chl a, Chl b and the carotenoid lutein, together with the structures of Chl a and Chl b. The spectrum of lutein is shown as an example of the carotenoid absorption range. Spectra of additional carotenoids: β-carotene, violaxanthin and neoxanthin are similar and show small red or blue-sifts respect with respect to lutein.*

Carotenoids (cars) absorb strongly in the blue-green (420-570nm) region of the light spectrum, but not in the Q region, therefore complementing the absorption spectrum in regions where Chl absorption is weak. The Cars include carotenes,

composed by carbon and hydrogen, while xanthophylls, also contain oxygen. Carotenes (such as β-carotene) are located in the RCs, while xanthophylls, including lutein (Lut), neoxanthin (Neo), violaxanthin (Vio) and zeaxanthin (Zea) are coordinated by antenna proteins. In most antenna proteins xanthophylls bind to four, conserved, binding sites called L1, L2, N1 and V1 in a ratio of 1:3 with Chlorophylls (Caffarri *et al.*, 2001; Croce *et al.*, 1999; Liu *et al.*, 2004). Although xanthophylls have generally been assumed to play a minor role in light-harvesting, it is becoming evident that they are more important than previously assumed (Collini, 2019). This is especially clear in diatoms, that contain Chl *c* instead of Chl *b* and complement their antennae complexes with huge amounts of Fucoxantin (Fx) (Wang *et al.*, 2019). Since Fx absorbs strongly in the blue-green light region, diatoms have a major advantage in aquatic environments where blue and green light penetrate much deeper than red light. Furthermore, the xanthophylls are especially important in photoprotection, as will be discussed later in this chapter.

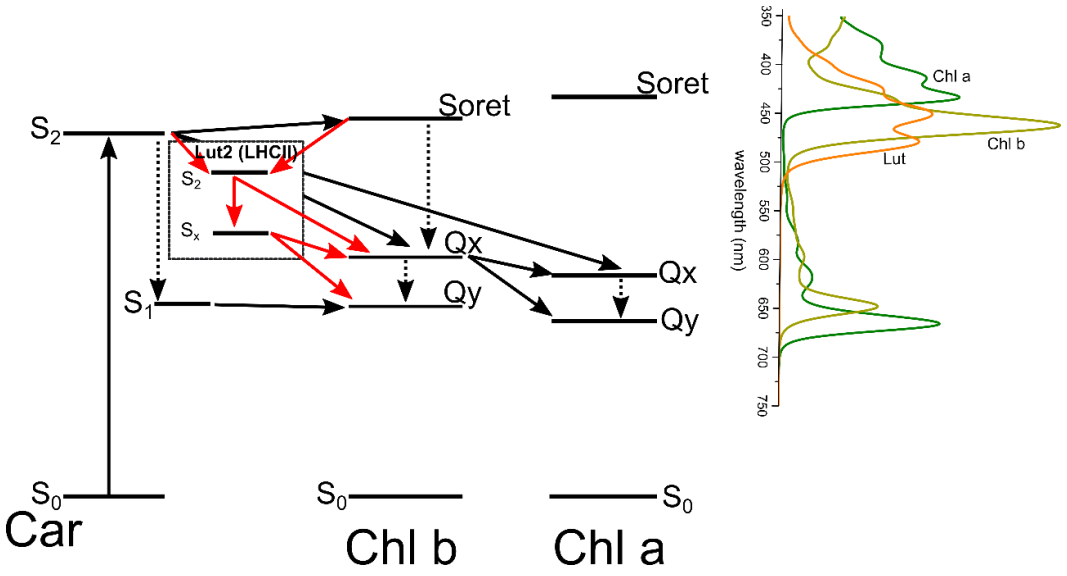


Figure 2. *Schematic of the energy levels of carotenoids and chlorophyll together with the absorption spectra of Chl a, Chl b and lutein. The black arrows indicate the original energy transfer found by Croce et al 2001, while the red arrows depict the newly found energy transfer in LHCII trimers Son et al 2019.*

Besides harvesting light, chromophores are essential for energy transfer of the absorbed photons. The excitons formed upon absorption by Chl or Cars are funnelled towards the RCs via an intricate organization of higher and lower energy

absorbing chlorophylls (Cinque *et al.*, 2000; Ramanan *et al.*, 2015). Chlorophylls undergo transitions to several excited states: those promoted by blue light (Soret-band) are in general very short-lived (ps) and fall back in fs to the lower excited states (Q-bands), accompanied by the release of heat, the Q-bands have a longer lifetime (ns), see Fig. 2. The transition of Cars from the ground state to the 1st excited singlet state (S_1) is optically forbidden and does not occur; however, the transition to the S_2 state is allowed and absorbs strongly in the blue-green region, but excitons quickly fall back to the S_1 state. Carotenoid excitations can be transferred to the Soret bands of Chl *b* or to the Q bands of both Chl *a* and Chl *b* (Croce *et al.*, 2001). Recently, an ultra-fast energy transfer from the Chl Soret bands or S_2 states of xanthophylls to the lower S_2 state of Lut2 in trimeric LHCII was detected. The energy in Lut2 can then be donated to an S_x-state (Son *et al.*, 2019). A similar process was previously identified in the bacterial LH2 complex (Ostroumov *et al.*, 2013). The S_x state in LHCII is unique to the Lutein binding site L2, due to the conformation imposed by the protein scaffold during trimerization (Liguori *et al.*, 2017; Son *et al.*, 2019). The S_x state serves as an efficient energy trap transferring to the lower Q_y and Q_x states of Chl (Son *et al.*, 2019); see Fig. 2.

The core complexes, RCI and RCII are very ‘expensive’ pigment-protein machines, costing a lot of energy to produce. As mentioned above it is possible to produce mutants with only the core complexes; however, they do not have enough chromophores to function efficiently in dim light, and in nature the core complexes are surrounded by other chromophore-binding proteins: i.e. the second moiety of photosystems, the “antenna system”, that funnel the excitations towards the RCs. The size of the antennae can easily be adjusted to the specific needs of the organism: in low light environments an organism will require more antenna complexes while high light conditions require smaller antennae complexes.

The core complexes of PSII and PSI

The PSII-core complex consists of least 20 subunits among which are the membrane intrinsic subunits D1 and D2 (binding the P680 special pair) and the two inner antenna proteins CP43 and CP47 (Umena *et al.*, 2011; Wei *et al.*, 2016; Su *et al.*, 2017), see Fig. 3. In total the PSII-core contains approximately 35 Chl *a* and 11 β-carotenes (Umena *et al.*, 2011), in-vivo it is always found as a dimer (Morris *et al.*, 1997; Santini *et al.*, 1994; Morosinotto *et al.*, 2006)

On the luminal side of the thylakoid membranes, the core complex of PSII harbours the oxygen evolving complex (OEC) comprised of PsbQ, PsbP and PsbO subunits and the Mn cluster (Mn_4CaO_5) which splits water into protons and oxygen to obtain electrons for the electron transfer (Wei *et al.*, 2016), see Fig. 3. The structure is very well conserved in all oxygenic photosynthetic organisms, from cyanobacteria to plants (Umena *et al.*, 2011; Wei *et al.*, 2016; Su *et al.*, 2017).

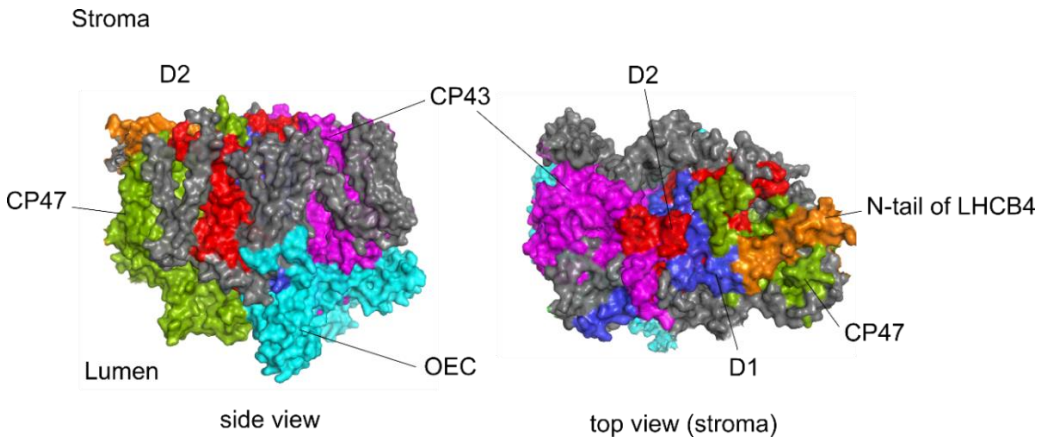


Figure 3. *Structure of the PSII-core complex; left shows the side view of the complex in the membranes and on the right is the top view from the stromal side. The Oxygen Evolving Complex as OEC (cyan), D1 (blue), D2 (red), CP43 (magenta), CP47 (green), the N-terminus tail of LHCB4 (orange). Structure from Su et al 2017, PDB-code 5XNM.*

A critical feature of PSII core complex is the scarcity of carotenoids, considering that PSII is the major target site of charge recombination and photoinhibition (Rutherford and Mullet, 1981) and carotenoids are antioxidants. Indeed, although carotenoids could, in principle, be photoprotective by quenching ^{3}Chl* issued from

charge recombination, they cannot sit in the spot of their maximal efficiency, i.e. near P680. This is because of the highly positive redox intermediates of Mn created by successive oxidation of P680 (approx. +1 volt) which would oxidize carotenes, causing rapid turnover of carotenes (Telfer, 2002). This makes PSII sensitive to photoinhibition from singlet oxygen and, at the same time, the trigger for the acclimative induction of photoprotective mechanisms. Even so carotenoids in PSII still conserve part of their photoprotective activity as singlet oxygen scavengers by a suicide mechanism, yielding carotenoid cleavage products among which β-cyclocitral, a major signalling product (Carmody *et al.*, 2016; D'Alessandro *et al.*, 2018). Indeed, carotenoid biosynthesis has a peculiar flux regime with β-carotene being synthesized at high rate and rapidly turned over from binding sites, while the synthesis rate of downstream xanthophylls bound to LHC antenna proteins is more than 20 times lower despite their high abundance (Beisel *et al.*, 2010). By contrast the antenna complexes of PSII have many carotenes, which are active and efficient in restricting the production of ROS species

The core complex of PSI is also highly conserved throughout evolution, apart for small changes in subunit compositions and the formation of multimeric complexes. Cyanobacterial PSI contains approximately 12 protein subunits of which the two subunits, PsaA and PsaB, form the core of the reaction centre (Mühlenhoff *et al.*, 1993; Jordan *et al.*, 2001) as well as acting as inner antenna complexes, similar to CP43 and CP47 of PSII to which they show homology (Cardona, 2017). Each PSI monomer contains a large inner antenna with 96 Chl *a* and 22 carotenoids besides the chromophores for electron transport, i.e. 3 Iron-Sulphur clusters and 2 phylloquinones (Jordan *et al.*, 2001). PSI can be found as either a trimer or a tetramer, depending on the cyanobacterial species (Jordan *et al.*, 2001; Watanabe *et al.*, 2014; Li *et al.*, 2014). The large chlorophyll complements of the PSI core complex imply it can function efficiently without external antenna complexes. Nevertheless, in higher plants and green algae PSI is only found as a monomer and is endowed with a multisubunit light harvesting complex called LHCI. The energy captured by LHCI is transferred to the RC in the core complex with an efficiency of almost 100% (Croce *et al.*, 2000). The plant PSI-core complex is composed of 14 subunit, of which several are unique to plants and green algae, such as PsaG,

PsaH, PsaN and PsaO, which have been shown to be involved in the binding of external antenna complexes (Scheller *et al.*, 2001; Knoetzel *et al.*, 2002; Amunts *et al.*, 2010), see Fig. 7. PsaG and PsaN are essential for the binding of LHCI, while PsaH and PsaO are essential for the binding of LHCII which may migrate to PSI during state-transitions(Lunde *et al.*, 2000; Jensen *et al.*, 2002; Varotto *et al.*, 2002; Amunts *et al.*, 2010; Ben-Shem *et al.*, 2003; Amunts *et al.*, 2007; Pan *et al.*, 2018).

The two photosystems differ slightly in their absorption maxima, the Chl dimer (P680) in PSII absorbs at 680nm, while the Chl pair (P700) in PSI has a maximum at 700nm. This difference in absorption peaks is important to decrease the overlap of their absorption bands and so to reduce competition for the same photons, allowing a balance of the energy absorption rate between the two PSs.

Light harvesting

If the core complexes of the two photosystems are very similar through many different organisms, the antenna complexes couldn't be more diverse. To understand this diversity of the different antennae it is important to follow the evolutionary origin of the LHC complexes. The oxygenic photosynthesis that can be found in eukaryotes these day, e.g. plants and algae, actually arose from one or more endosymbiotic events, in which an eukaryote cell incorporated a cyanobacterial ancestor (Margulis, 1981; Blankenship, 2010; Jensen and Leister, 2014; Keeling, 2010). Integration of these prokaryotes with the host cell included transfer of genes to the nuclear genome, ultimately leading to plastids, such as cyanelles, rhodoplasts and chloroplasts, that we currently find in the glaucophytes, red and green algae, respectively (Jensen and Leister, 2014; Keeling, 2010). The plastids have undergone a lot of changes from the initial cyanobacteria (Jensen and Leister, 2014; Moreira *et al.*, 2000). Differences among plastid types can be found in the antenna proteins: cyanobacteria use phycobilisomes for light harvesting and High-Light Inducible Proteins (HLIPs) for photoprotection, while green algae and plants rely on light harvesting complexes (LHCs) for both functions. Together with the development of different antenna systems a difference in chromophore

composition emerged. Besides Chl a, which is found in all the algae species; different types of Chl can be found in different taxa.

In green algae and plants, LHCs are encoded by the multigenic *Lhc*-family (Jansson, 1999). These antenna proteins connect to either the PSII-core (LHCB) or the PSI-core (LHCA). As mentioned above, these proteins bind Chl *a*, Chl *b* and carotenoids. The LHC-proteins have a similar structure that comprises three α-helices spanning the membrane (Liu *et al.*, 2004; Ben-Shem *et al.*, 2003). They most likely evolved from the HLIPs found in cyanobacteria (Dolganov *et al.*, 1995), which bind Chl *a* and β-carotene but only contain one transmembrane helix (Knoppová *et al.*, 2014; Staleva *et al.*, 2015). HLIPs have been found to be involved in many different functions, such as light acclimation (Havaux *et al.*, 2003), Singlet Oxygen Scavenging (Sinha *et al.*, 2012), protection of assembly intermediated during biosynthesis of Chl-binding proteins (Chidgey *et al.*, 2014) and the regulation and recycling of Chls (Xu *et al.*, 2002; Vavilin and Vermaas, 2002; Hernandez-Prieto *et al.*, 2011; Vavilin *et al.*, 2007). Light-harvesting, thus, doesn't seem to be one of original functions of ancestral LHCs and yet modern LHCs are now the most abundant membrane proteins on the Earth. Possibly because the changes, which made them light harvesters, are built over an ancestral photoprotection-efficient architecture which is crucial when colonizing a challenging environment such as land. Thus, light harvesting proteins of green algae and higher plants appear in a number of variants among which several have crucial photoprotective roles.

In green algae and plants each half of the dimeric PSII-core binds one copy of the monomeric antenna proteins LHCB4 (CP29) and LHCB5 (CP26), see Fig. 4. The double naming of these antenna proteins can be confusing since they are used interchangeably. The original names, from the '80s of last century are descriptive and made out of combining "CP" which stands for: Chlorophyll-Protein (CP) with the molecular weight of the respective holoprotein in green gels (Bassi *et al.*, 2018). Later, when the complexity of genes encoding pigment-binding proteins was recognized, the fact that the green bands belonged to the common Light Harvesting Complex family was acknowledged by the "LHC" notation (*Lhc* for genes),

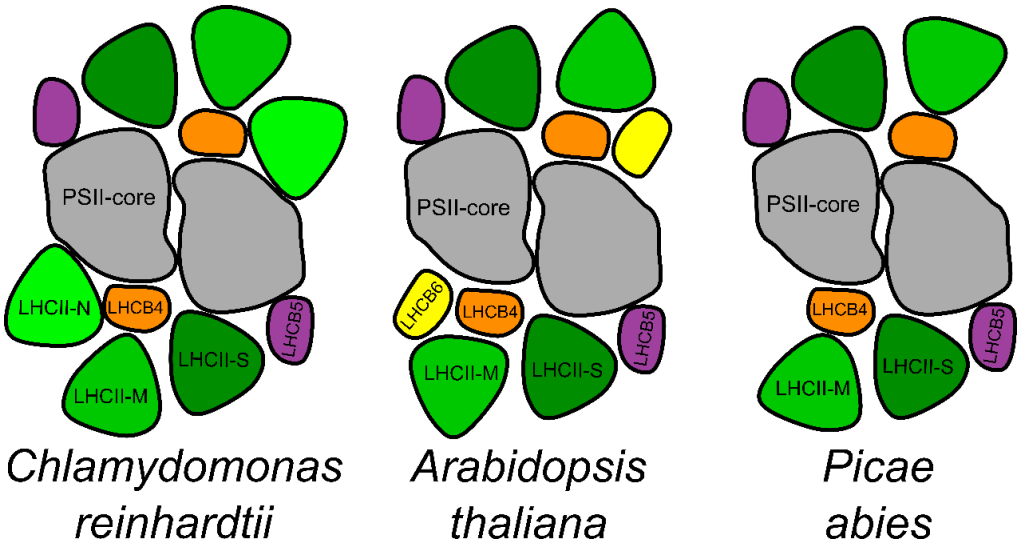


Figure 4. *Schematic over of the PSII-supercomplexes in different organisms C. reinhardtii, A. thaliana and P. abies. PSII-core (grey), LHCB4 (orange), LHCB5 (purple), LHCB6 (yellow), LHCII trimers S, M and N (in green). Schematic is based on the electron microscopy pictures of Kouril et al 2016.*

followed by the letter 'a' or 'b' for their connection to either PSI or PSII respectively (Jansson *et al.*, 1992). Even though this has worked well for most of the antenna proteins, LHCB4 and LHCB5 are often still indicated by CP29 and CP26 respectively, which ignores the fact that CP29 is encoded by three different genes with very similar properties (de Bianchi *et al.*, 2008).

LHCB4 (CP29) is located between LHCII-M (moderately bound) and the core of PSII with which it interacts at the internal antenna protein CP47 (Tokutsu *et al.*, 2012; Drop *et al.*, 2014; Yakushevska *et al.*, 2003; Su *et al.*, 2017; Egbert J. Boekema *et al.*, 1999), see Fig. 5. LHCB4 couples excitation energy transfer between the PSII-core and the outer antenna complexes (Caffarri *et al.*, 2011) and contains 13-14 chlorophylls (10 Chl *a* and 3-4 Chl *b*), a Lutein, Neoxanthin and Violaxanthin (Pan *et al.*, 2011; Wei *et al.*, 2016). One additional interactor of LHCB4, in plants but not in algae, is LHCB6 (CP24) which coordinates additional LHCII-L (loosely bound) trimers accumulating in low light conditions (Ballottari *et al.*, 2007). Finally, LHCB4 is an interacting partner of PSBS, the pH sensor

protein essential for triggering Non-Photochemical Quenching (NPQ) (Dall'Osto *et al.*, 2017).

LHCB5 (CP26) is bound to CP43, the other internal antenna protein of the PSII-core, together with the LHCII-S trimer (strongly bound) that is also interacting with LHCB4, see Fig. 5. Lhcb5 probably plays a role in the excitation energy transfer (EET) from LHCII to CP43, although less critical with respect to the Energy Transfer (ET) from LHCB4 to CP47 due to the presence of an alternative ET pathway directly from LHCII-S to CP43 (Caffarri *et al.*, 2011; Wei *et al.*, 2016). LHCB5 contains 13 chlorophylls (8-9 Chl a and 4-5 Chl b), 1-2 Lutein, 0-1 Violaxanthin and 1 Neoxanthin (Ballottari *et al.*, 2009; Wei *et al.*, 2016). LHCB5 bind Zeaxanthin upon high light exposure and this appears to be a major factor in the qZ component of NPQ (Dall'Osto *et al.*, 2005), see below.

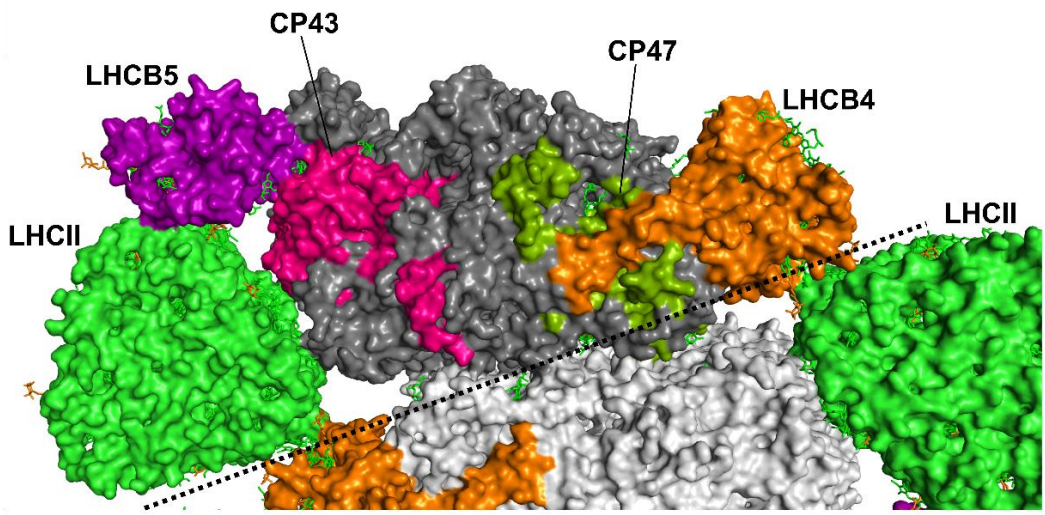


Figure 5. *Structure of the PSII-core complex (grey) with the major light harvesting complex LHCII (light green) and the minor complexes LHCB4 (orange) and LHCB5 (purple) attached to CP47 (dark green) and CP43 (violet), respectively. Structure from Su et al 2017, PDB code 5XNM*

LHCII forms trimers in a homo- or heterotrimeric composition. Each LHCII monomer binds 8 chlorophylls *a* (Chl *a*), 6 chlorophylls *b* (Chl *b*) and 4 carotenoids; 2 Lutein, 1 Neoxanthin and 1 Violaxanthin or Zeaxanthin (Liu *et al.*, 2004). The carotenoids are attached to binding sites named V, L1, L2 and N1, respectively.

While L1, L2 and N1 are active in ET to Chl *a*, V1 is not (Caffarri *et al.*, 2001) and the Violaxanthin ligand is exchanged to Zeaxanthin in HL. Although trimers are found in all the members of the green clade, they are more stable in higher plants. Trimerization confers special properties to LHCII by tuning its absorption spectrum: the Q_y transition of Chl *b* is enhanced and that of Chl *a* is red shifted (Peterman *et al.*, 1997). Even more important is the twisting of Lutein in binding site 2 causing a red-shift of the Lut2 S_1 state which makes it the hub for excitation delivery from cars to Chl a (Son *et al.*, 2019). The ET from LHCII to the PSII-core occurs via the monomeric antennas (LHCB4 and LHCB5), while direct energy transfer from LHCII to PSII core is far less efficient as shown by the mutant lacking all monomeric antenna complexes (van Oort *et al.*, 2010; Dall'Osto *et al.*, 2017). Besides playing a major role in energy transfer to the core, both LHCB4 and LHCB5 are important in the proper organisation and stability of LHCII with the PSII-core (Yakushevska *et al.*, 2003; de Bianchi *et al.*, 2011).

In *Arabidopsis thaliana* LHCII is encoded by 9 genes, belonging to three groups (*Lhcb1-3*), LHCB1 and LHCB2 can form homotrimers or heterotrimers, while LHCB3 is only found in heterotrimeric complexes. Even though the sequences of the monomeric LHCIIs are very similar, they have different roles in regulation of energy transfer. LHCB1, the most abundant isoform, is necessary for NPQ (Pietrzykowska *et al.*, 2014), while LHCB2 is essential for state 1-2 transitions (Leoni *et al.*, 2013). A third type of LHCII protein, LHCB3 is even less abundant and its deletion has not shown a specific functional phenotype, so far (Damkjær *et al.*, 2009).

In the green alga *C. reinhardtii* LHCII is also encoded by 9 genes but there is no correspondence with the *Lhcb* genes in *A. thaliana* indicating these evolved after the divergence of *C. reinhardtii* from the green lineage (Ballottari *et al.*, 2012). The genes found in *C. reinhardtii* were named (*Lhcbm1-9*) and can be divided into 4 different groups based on their sequence identity (Minagawa and Takahashi, 2004; Teramoto *et al.*, 2001).

Table 1. *Different PSII antenna proteins found in C. reinhardtii and their respective group. Minagawa and Takahashi, 2004 and Teramoto et al., 2001*

Type I	LHCBM3, 4, 6, 8 and 9
Type II	LHCBM5
Type III	LHCBM2 and 7
Type IV	LHCBM1

The sequence identity in each group is high, while the averaged sequence identities between LHCBM6 (Type I) and Type II, Type III or Type IV is respectively 80%, 77% or 74% (Minagawa and Takahashi, 2004), see Fig. 6. As is the case in plants, the different types of monomers in LHCII have different functions.

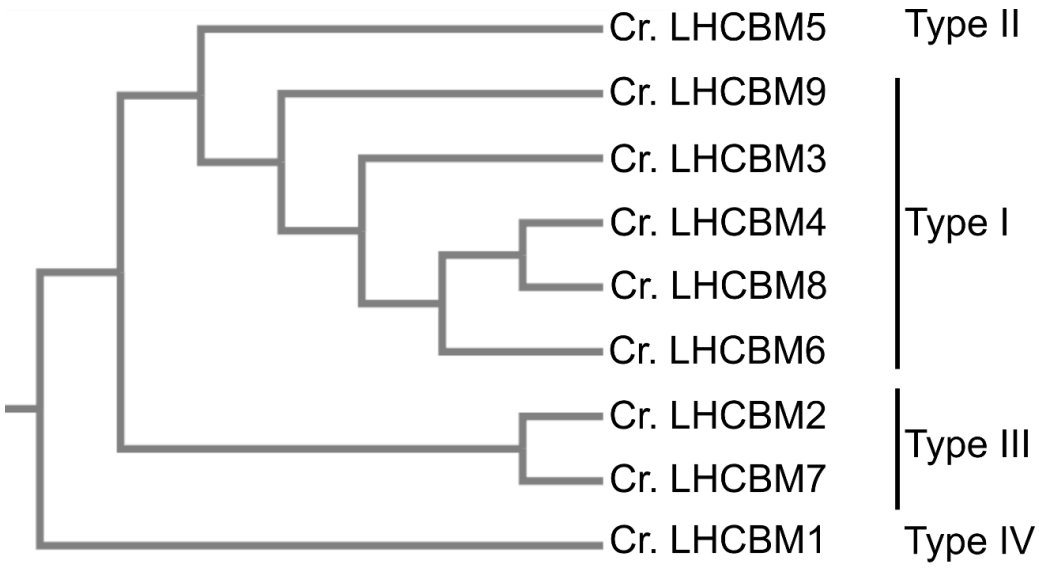


Figure 6. *Phylogenetic tree of the PSII antenna proteins found in C. reinhardtii*

Type I (LHCBM3, 4, 6, 8 and 9).

LHCBM4, 6 and 8 were primarily found as 'free' LHCIIs not connected to either photosystem (Girolomoni *et al.*, 2016), suggesting to be LHCII-only domains that are most likely involved in state-transitions (Girolomoni *et al.*, 2016; Nagy *et al.*,

2014; Ünlü *et al.*, 2014). Knock-down of these subunits significantly reduced the capacity to perform state 1-2 transitions, the total LHCII content and the capacity to perform NPQ (Girolomoni *et al.*, 2016).

LHCBM3, being one of the more abundant LHCII subunits, is mainly bound as heterotrimers to PSII (Drop *et al.*, 2014). The precise role of LHCBM3 however remains unclear, although it is possible that it has no other specific role besides light harvesting.

LHCBM9 is only found with very low abundance during normal growth conditions; however, it is strongly up-regulated during a variety of stress conditions, such as sulphur starvation or anaerobic growth (Nguyen *et al.*, 2008; Grewe *et al.*, 2014; González-Ballester *et al.*, 2010; Sawyer *et al.*, 2015). During up-regulation ofLHCBM, other LHCBMs are replaced and the LHCII complexes reduced their fluorescence yield. Furthermore, PSII showed an increased dissipative state and an overall reduction of Reactive Oxygen Species (ROS) was observed, indicating that LHCBM9 is involved in photoprotection only during specific stress conditions (Grewe *et al.*, 2014).

Type II (LHCBM5)

LHCBM5 is most likely involved in state transitions and are mainly found in the 'free' LHCIIs like LHCBM4, 6 and 8 (Drop *et al.*, 2014). Indicating that LHCBM5 is most likely involved in state-transitions, which is consistent with the fact that besides LHCBM5, LHCBM4, LHCBM6 and LHCBM8 can be phosphorylated by the Stt7 kinase, a protein essential for state 1-2 transitions (Takahashi *et al.*, 2006; Lemeille *et al.*, 2009).

Type III (LHCBM2 and 7)

LHCBM2 and LHCBM7, although slightly different in gene sequence, they produce exactly the same mature amino acid sequence (Elrad and Grossman, 2004; Stauber *et al.*, 2003). Together with LHCBM3, LHCBM2 and LHCBM7, this group makes up most of the LHCII-trimers and silencing of *Lhcbm2* and *Lhcbm7* genes in *C. reinhardtii,* therefore resulta in a decreased LHCII abundance (Ferrante *et al.*, 2012). Furthermore, these mutants showed an increased sensitivity to superoxide

anions, probably due to a lower level of neoxanthin in which LHCBM2/7 are most likely enriched and which is involved in superoxide anion scavenging (Dall'Osto *et al.*, 2007; Ferrante *et al.*, 2012). Besides the sensitivity to superoxide anions, the ability to perform state transitions was decreased with the absence of LHCBM2 and LHCBM7 (Ferrante *et al.*, 2012).

Type IV (LHCBM1)

LHCBM1 is the most highly abundant subunit in *C. reinhardtii* and it has been shown to be involved in NPQ. Absence of LHCBM1 leads to increased sensitivity to superoxide anions and singlet oxygen (Elrad *et al.*, 2002; Ferrante *et al.*, 2012). The sensitivity to superoxide anions is most likely related to a decreased level of neoxanthin, as observed in the *Lhcbm2/7* knock-downs. The reduced capacity of scavenging singlet oxygen is probably due to the location of LHCBM1, which is close to the reaction centre, where a lot of singlet oxygen is generally produced by charge recombination (Ferrante *et al.*, 2012).

The PSII structure of flowering plants (angiosperms) is different in comparison to algae, because it contains a third monomeric protein named LHCB6 (CP24) which is unique to land plants and was found to be essential for the energy transfer to the core of PSII (Alboresi *et al.*, 2008; van Oort *et al.*, 2010). LHCB6 forms a stable dimer with LHCB4 (Su *et al.*, 2017) and replaces the LHCII-N trimer, see Fig. 3. In the absence of LHCB4, LHCB6 is not expressed and a different supercomplex organisation was observed (de Bianchi *et al.*, 2011). LHCB3 is another protein unique to land plants and belongs to the LHCII-M trimer (Alboresi *et al.*, 2008; Dainese and Bassi, 1991) and, together with LHCB6, is essential for the stable binding of the LHCII-M trimer to PSII (Caffarri *et al.*, 2009; Kovács *et al.*, 2006; Kouřil *et al.*, 2013). It was generally believed that these proteins were crucial for the transition from the aquatic life to terrestrial; however, it was found that subgroups of gymnosperms lost functional LHCB6 and LHCB3 during evolution, thus leading to a PSII supercomplex structure very similar to that found in algae (Kouřil *et al.*, 2016). LHCB6 and LHCB3 are probably involved in long- and short-term light acclimation, since angiosperms adapted to high light conditions reduces

the amount of these proteins, effectively decreasing the size of the PSII supercomplex (Kouřil *et al.*, 2013).

Antenna complexes of PSI

In the case of green algae and higher plants, the LHC-type antenna system is named Light Harvesting Complex I (LHCI). In higher plants the basic form of LHCI consists of two heterodimers, LHCA1/LHCA4 and LHCA2/LHCA3, which form a belt on one side of the PSI-core (Ben-Shem *et al.*, 2003). These two heterodimers are very similar to the heterodimer of LHCB4/LHCB6 of PSII in plants (Su *et al.*, 2017). Plant LHCA1-LHCA4 bind in total 45 Chl *a*, 12 Chl *b* and 13 carotenoids (Qin *et al.*, 2015). Chl *b* is mainly bound to LHCA4 and LHCA2 which are located slightly further from the core of PSI (see Fig. 7) and most of the energy harvested by LHCI likely travels via LHCA1 and LHCA3 to PsaB and PsaA, respectively

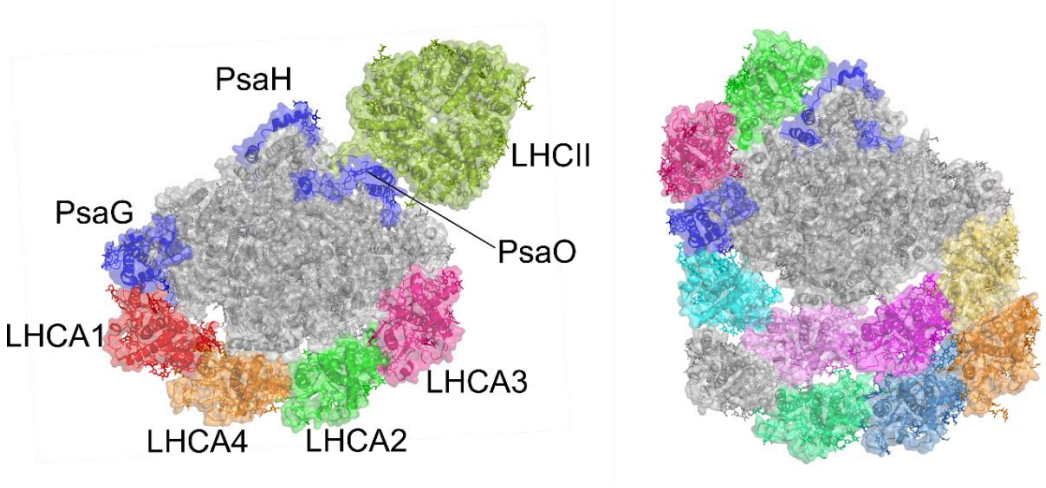


Figure 7. Left: *Crystal structure of maize PSI with LHCII attached. Structure from Pan et al 2018, PDB code 5JZI. Right: Crystal structure of PSI-LHCI from Bryopsis corticulans. Structure from Qin et al 2019, PDB code 6IGZ.*

(Qin *et al.*, 2015; Mazor *et al.*, 2017).

Despite the large size, this PSI-LHCI complex performs photochemistry with a quantum efficiency close to 1, meaning that it is able to produce an electron for almost every absorbed photon, thus making PSI the most efficient photochemical

energy converter (Nelson, 2009; Croce and van Amerongen, 2013). Despite the well-defined location of LHCI-subunits, two additional *Lhca* genes are expressed in *A. thaliana*: the corresponding LHCA5 and LHCA6 proteins were located in large and rare PSI-NDH complexes catalysing cyclic electron transport (Peng *et al.*, 2009).

The distribution of antenna subunit within the PSI-LHCI in green algae has been long debated, but a recent 3D structure seems to shed light on it (Qin *et al.*, 2019). The 10 LHCI subunits (Bassi *et al.*, 1992) are distributed into two different locations of the supercomplex. Eight form a double moon shaped arc with 4 LHCA proteins each while two additional LHCA subunits are bound opposite to the rest of LHCIs in between PsaH and PsaG, see Fig. 7. The PSI-LHCI supercomplex in *C. reinhardtii* contains less 'red forms' and more Chl b in comparison to plants, which leads to a blue-shift in the fluorescence emission spectrum 705nm vs 730nm (Drop *et al.*, 2011; Bassi and Simpson, 1987). This can be explained by the fact that algae live in water, where the blue light penetrates best. Water absorbs far red light and thus is a very poor energy source for aquatic photosynthetic organisms.

Besides LHCI, LHCII is also known to bind to PSI in order to balance the energy distribution between the two photosystems. Beside LHCII binding to PSI upon transition to state 2, PSI complexes were found to bind LHCII-trimers in steady-state illumination conditions (Wientjes *et al.*, 2013). The binding of LHCII to PSI, increases the total amount of chlorophylls by approximately 20% with a minimal quantum efficiency loss of 0.2% (Wientjes *et al.*, 2013). Furthermore, it simplifies the acclimation to different light intensities, where the antenna size has to be increased or decreased, because only LHCB1 and LHCB2 have to be regulated (Wientjes *et al.*, 2013; Ballottari *et al.*, 2007).

In green algae, besides LHCII, the monomeric antenna complexes, LHCB4 and LHCB5, are also known to migrate from PSII to PSI during state 1-2 transitions, forming an even larger complex in comparison to the one found in plants (Kargul *et al.*, 2005; Takahashi *et al.*, 2006; Tokutsu *et al.*, 2009; Drop *et al.*, 2014).

Fucoxanthin Chlorophyll binding Proteins

The core complexes of diatoms are very similar to that found in green algae and form a monomer and dimer for PSI and PSII, respectively (Ikeda *et al.*, 2013; Veith and Büchel, 2007; Nagao *et al.*, 2007). However, much remains unknown about the lateral distribution of the supercomplexes in the thylakoid membrane. The crystal structure of a dimeric Fucoxanthin Chlorophyll binding Protein (FCP) was reported to show that the LHC monomer binds 7 Chl *a*, 2 Chl *c*, 7 Fucoxanthin and 1 Diadinoxanthin (Wang *et al.*, 2019), see Fig. 8. The FCP has a Car/Chl ratio close to 1, much higher than in green algae.

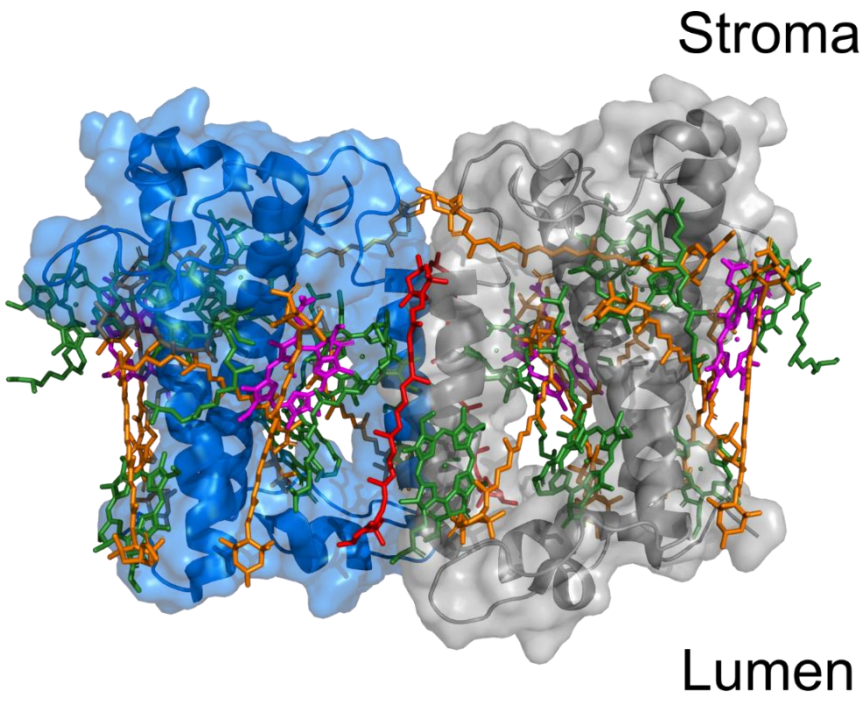


Figure 8. *Dimeric FCP in blue and grey. With Chl a (green), Chl c (magenta), Fx (orange) and diadinoxanthin (red). Structure from Wang et al 2019, PDB-code: 6a2w.*

The backbone structure of the transmembrane helices of the FCP monomers is similar to that of the LHCs found in green algae and plants (Wang *et al.*, 2019). Out of the four xanthophyll-binding sites of plant LHCII only two are conserved in FCP, which correspond to L1 and L2 sites, both hosting Fucoxanthin in FCP (Wang *et al.*, 2019). The V1 and N1 binding sites of LHCII are not conserved in FCP, while

5 new Fucoxanthin binding sites are distributed at the periphery of the complex either in a transmembrane arrangement or parallel to the stromal surface. Finally, two Diadinoxanthin binding sites are located at the dimer interface (Wang *et al.*, 2019). In total six of the nine Chls (four Chl *a* and two Chl *c*) are found in similar positions to LHCII, while the remaining three Chls *a* are bound in new positions.

Each Fucoxanthin chromophore is closely associated to one or more Chls to allow for fast energy transfer from Fucoxanthin to Chl *a* (Papagiannakis *et al.*, 2005; Akimoto *et al.*, 2014; Gelzinis *et al.*, 2015; Wang *et al.*, 2019). Two Fucoxanthin molecules, Fx306 and Fx307, are not in close contact with any Chl *a* and likely transfer energy to neighbouring Chl *c*'s (Wang *et al.*, 2019), which, in turn, efficiently transfer to Chl *a* (Gelzinis *et al.*, 2015). Due to the high density of xanthophylls in the structure, it cannot be excluded that energy transfer occurs between the xanthophylls, like that observed from Lut1 to Lut2 in LHCII-trimers (Son *et al.*, 2019). Besides an efficient energy transfer from Fucoxanthin to Chl *a*, the close proximity of the two species might allow a transfer from Chl to Fucoxanthin in excess light conditions (Goss and Lepetit, 2015).

Photoprotection

Photosynthetic organisms must deal with an extreme variability of light: at sunrise light is limiting and fully used for photochemistry to fuel the electron transport. At midday, however, light can be too much, and the RCs are not able to quench all the singlet Chl excited states (^{1}Chl*) generated by an oversupply of photon absorption. Thus, the excitations remain longer on the Chls, which gives the possibility to intersystem crossing and the production of Chl triplet states (^{3}Chl*). ^{3}Chl* are dangerous because they can react with O_2 and create singlet oxygen ($^1O_2$*), see Fig. 9.

The chlorophyll dimer in the RC of PSII is also prone to the production of $^1O_2$* due to the increased chance of creating ^{3}Chl* states upon charge recombination (Durrant *et al.*, 1990). On the other hand, the presence of many carotenes on the LHCs provides for the quenching of high energy states without the harmful side reactions

with oxygen. Protective strategies include scavenging of ROS (Asada, 1999), prevention of the formation of ROS by quenching ^{3}Chl* (Dall'Osto *et al.*, 2012) and quenching of the excess ^{1}Chl* through Non-Photochemical Quenching (NPQ). The half-life of singlet oxygen is 200ns and it has been calculated to diffuse approximately 10nm in physiological tissue (Gorman and Rodgers, 1992; Sies and Menck, 1992), meaning that the damage occurs in the place where the singlet oxygen is produced. While photosynthetic organisms have developed several strategies to counteract these negative by-products, the D1 subunit of PSII is still a prime target for ROS production and is the most turned-over protein in photosynthesis.

Nevertheless, photoprotective strategies have evolved to lessen damage to PSII. Of all the photoprotective strategies, the most dynamic process, NPQ, catalyses dissipation of excess energy as heat, thereby introducing an alternative pathway to shorten the lifetime of the ^{1}Chl* and thus compete with ^{3}Chl* formation. NPQ rapidly (within seconds) reacts to the increased levels of the ^{1}Chl* and dissipates excess excitation energy as heat (Genty *et al.*, 1989; Müller *et al.*, 2001).

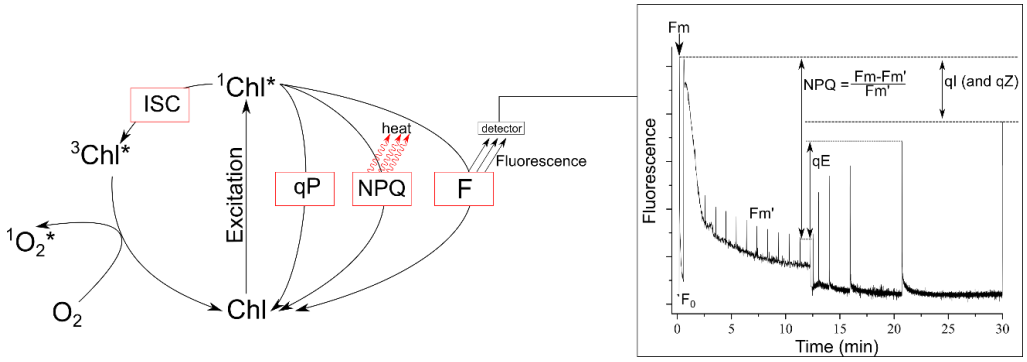


Figure 9. *Schematic of the different energy dissipation pathways of chlorophylls. Chlorophylls are excited by photons and transfer the excitation energy to the RCs where they are used for photochemistry (qP). In high light conditions the RCs are completely occupied, and the excited chlorophylls can follow two pathways, i) the formation of chlorophyll triplets via Inter System Crossing (ISC) and reaction with oxygen leading to ROS formation or ii) excitations are dissipated has heat by Non-Photochemical Quenching (NPQ). A part of the excited states returns to the ground state by re-emitting a photon (fluorescence) which can be measured*

by chlorophyll fluorescence and gives information on the different aspects of NPQ such as energy dissipation (qE) and photoinhibition (qI). F_0 is the basal fluorescence, while Fm is the highest fluorescence after a saturating light pulse in dark adapted tissue. Saturating pulses are short enough not to activate NPQ, but ensure that PSII-RCs are closed. After measurement of Fm, actinic light is switched on and fluorescence drops due to the activation of NPQ mechanisms. Saturating light pulses give Fm', with which NPQ can be calculated.

NPQ is triggered by the low pH generated in the thylakoid lumen due to the inhibition of ATPase activity in excess light conditions. Indeed, when the rate of the CO_2 reducing Calvin-Benson cycle is saturated, ADP+Pi cannot regenerate thus inhibiting ATPase activity by lack of substrate and, consequently, the return of H^+, accumulated by water splitting and the Q-cycle in the stromal compartment. Also, NPQ requires special members of the LHC-protein family for its activation. Besides these light harvesting functions for PSII and PSI, LHCs also catalyse energy quenching through a reorganization of the interactions between chromophores either elicited by direct protonation of lumen-exposed acidic residues as in the case of LHCSR proteins (Ballottari *et al.*, 2016) or caused by interactions with PSBS (Betterle *et al.*, 2009), a proton sensitive protein itself. LHCSR (Light-Harvesting Complex Stress Related) and PSBS (Photosystem II subunit S), are indispensable for NPQ respectively in green algae (Peers *et al.*, 2009) and vascular plants (Li *et al.*, 2000) together with the xanthophylls Lut and/or Zea which are ligands for LHC proteins (Polle *et al.*, 2001; Pogson *et al.*, 1998; K K Niyogi *et al.*, 2001). Interesting is the case of mosses, evolutionary intermediates between green algae and vascular plants, in which both PSBS and LHCSR proteins are active and trigger NPQ (Alboresi *et al.*, 2010).

The low lumenal pH also activates the protein violaxanthin de-epoxidase (VDE) which increases the length of the conjugated bonds of Violaxanthin in two subsequent steps creating first antheraxanthin and then Zeaxanthin (Demmig-Adams and Adams, 1996). While Violaxanthin is involved in light-harvesting and is able to transfer it's excitation energy to Chl *a* (Arnoux *et al.*, 2009; Owens *et al.*, 1987; Peterman *et al.*, 1997), the de-epoxidation increases the length of the

conjugated bonds, thereby lowering the S_1 state below that of the Q_y band of the Chl and making Zeaxanthin an excellent energy trap for Chl excitations (Frank *et al.*, 1994), see Fig. 2.

Zeaxanthin induces photoprotection in two ways; i) it is effective in scavenging ROS because it is a very strong antioxidant (Havaux *et al.*, 2007; Krinsky, 1979; Edge *et al.*, 1997) and ii) it enhances energy dissipation as heat (Niyogi *et al.*, 1998).

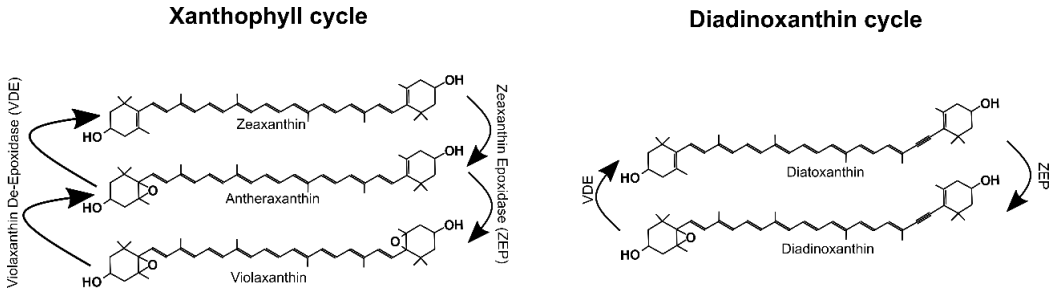


Figure 10. *The Violaxanthin cycle, generally referred to as the Xanthophyll cycle found in green algae and higher plants and the Diadinoxanthin cycle found in diatoms, dinophytes and haptophytes.*

The NPQ induced by the conversion of Vio into Zea, also referred to as the xanthophyll cycle (Fig. 10), is named qZ and conversion generally takes a few minutes. Relaxation of qZ, the conversion of Zea back into Vio by zeaxanthin epoxidase (ZEP), is far slower and can easily take up hours or even days if the organism experiences other stress factors. Similar to the xanthophyll cycle found in algae and plants, is the diadinoxanthin cycle found in diatoms, dinophytes and haptophytes, where diadinoxanthin is directly converted into diatoxanthin (Hager and Stransky, 1970), see Fig. 10. The proteins catalysing these reactions are very similar to VDE and ZEP, found in plants and green algae and some of these proteins are even able to convert both Violaxanthin and Diadinoxanthin into Zeaxanthin and Diatoxanthin respectively (Jakob *et al.*, 2001). The main difference between VDEs of plants vs diatoms is the pH sensitivity. The de-epoxidases found in diatoms are already activated at a pH of 7.2, while the activity of VDE in higher plants requires a pH lower than 6.5 (Jakob *et al.*, 2001) consistent with their reactivity to excess light. It is clear that in Chromealveolates (Chl *c*-containing algae) the equivalent of a Zeaxanthin Epoxidase (ZEP), forming diadinoxanthin from diatoxanthin, has

been inherited from a red algal/green algal ancestor and that this has led not only to NPQ under high light by a Diadinoxanthin Epoxidase but also to the evolution of the special allenoic and acetylenic xanthophylls such as fucoxanthin, peridinin and vaucheriaxanthin, which are such a notable feature of diatoms and allied algae (Dautermann and Lohr, 2017).

Due to the slow relaxation, qZ, is sometimes also confused with qI, the third component of NPQ which comprises photoinhibition and repair of damaged PSII. However, photoinhibition is caused by excess light and usually takes days before it relaxes, e.g. before all damaged proteins and chromophores have been repaired or replaced. It is not easy to distinguish qZ from qI without the use of inhibitors interfering either with the xanthophyll cycle (DTT) or D1 synthesis (chloramphenicol).

Another photoprotective mechanism of higher plants is (qM), i.e. the movement of chloroplasts to avoid excess light. Despite the fact that it induces a decrease in fluorescence, similar to qE or qZ, qM is not a genuine quenching process. Indeed, it relies on the decrease of the photon absorption when chloroplasts move away from direct light and align along the cell walls parallel to the incident light (Kasahara *et al.*, 2002; Dall'Osto *et al.*, 2014; Cazzaniga *et al.*, 2013). Interestingly, qM is mediated by phot2, a blue light photoreceptor. Consistently, the phot mutation in unicellular algae makes them incapable of swimming away from excess light (Trippens *et al.*, 2012).

qT, the quenching obtained when LHCII disconnects from long lifetime fluorescent PSII to connect with short lifetime fluorescent PSI, being quenched in the process, was found to be more pronounced in green algae, such as *C. reinhardtii*, than in plants (Kargul *et al.*, 2005; Takahashi *et al.*, 2006; Iwai *et al.*, 2008). It is the re-distribution of antenna complexes, also known as State Transitions (Allorent *et al.*, 2013).

The biophysical mechanisms of energy dissipation have long been discussed and currently three main hypotheses are supported that all include the role of establishing new interactions between two or more chromophores within LHC proteins in response to thylakoid lumen acidification, binding of xanthophyll cycle

pigments to allosteric sites or both. The first model was based on observations on isolated LHCII oligomers upon aggregation (Müller *et al.*, 2010), where a charge transfer state was observed involving excitonic coupling between Chls. This model does not directly implicate carotenoids, like Lutein and Zeaxanthin in quenching reactions despite their crucial importance for NPQ *in-vivo* (Krishna K. Niyogi *et al.*, 2001; Gilmore and Yamamoto, 1993), thus considering these act as allosteric modulators of quenching proteins. Two other theories, instead, propose interactions between xanthophylls and Chls to be directly involved in quenching, but differ in the mode of these reactions: Holt et al. 2005 and Ahn et al. 2008 proposed quenching is initiated by the formation of Chl dimers within LHCB4 yielding low lying states accepting electrons from excited Zeaxanthin to form a transient Zea^{+}/Chl^{-} radical cation (Holt *et al.*, 2005; Avenson *et al.*, 2008). Charge recombination to the ground state then, produces heat dissipation of excited states. A third and simpler model involves LHCII aggregation within the thylakoid membrane as a consequence of a decreased pH in the lumen, which brings Chl and Lutein chromophores in closer contact to each other thus allowing energy transfer from Chl *a* to Lut1 followed by rapid decay to the ground of the short-lived S_1 state (Ruban *et al.*, 2007). Similar to the case of model 1, Zeaxanthin is not considered as a chromophore directly involved in the quenching reaction(s), but rather an allosteric modulator.

Triggers of quenching reactions

Genetic analysis identified two proteins, PSBS and LHCSR whose deletion completely impairs the triggering of qE in excess light, respectively in pants and algae (Li *et al.*, 2000; Peers *et al.*, 2009).

PSBS, a four-helix protein, with homology to LHCs that does not bind pigments (Bonente *et al.*, 2008; Fan *et al.*, 2015; Peers *et al.*, 2009). Thus, PSBS cannot be the actual site of quenching. However, PSBS carries two lumen-exposed acidic residues whose mutation fully impairs quenching (Li *et al.*, 2004). Quenching reactions are therefore proposed to occur in interacting pigment-binding proteins among components of LHC antenna upon conformational changes consequent to

interaction with PSBS. Two distinctive sites have been identified (Holzwarth *et al.*, 2009): the monomeric antenna complexes (Ahn *et al.*, 2008; de Bianchi *et al.*, 2011) and LHCII (Ruban *et al.*, 2007; Dall'Osto *et al.*, 2017). Whether the PSBS-LHC interaction is direct (Ahn *et al.*, 2008) or is a consequence of membrane reorganization (Betterle *et al.*, 2009) is a matter of a lively debate.

In vivo measurements using WT mutants completely devoid of the monomeric antenna proteins LHCB4-6 (NoM) (Dall'Osto *et al.*, 2017) showed that PSBS most likely interacts at two different sites, the first and fastest activated is the monomeric antenna complex LHCB4 where it induces a conformational change that not only increases the exchange rate of Violaxanthin to Zeaxanthin (Morosinotto *et al.*, 2002) but also increases the interactions between Chl *a* and Lutein (Li *et al.*, 2009) or Zeaxanthin (Avenson *et al.*, 2008) and between two Chl *a* molecules (Ahn *et al.*, 2008). The formation of a radical cation was detected in the WT plants, but not in the NoM mutant lacking the monomeric antenna LHCB4-6, suggesting that the quenching reaction is accompanied by the formation of either a Zeaxanthin or Lutein radical cation (Dall'Osto *et al.*, 2017) and yet residual quenching is still active, involving PSBS and LHCII. Thus, it appears that quenching is a twofold process involving LHCB4 and PSBS to form the carotenoid cation ($Car^{\circ+}$) on one hand and LHCII and PSBS to form an ET from Chl to Zeaxanthin, followed by fast decay to the ground state (Dall'Osto *et al.*, 2017).

LHCSR is strikingly different from PSBS by the fact that it binds Chl *a*, Lutein and Violaxanthin, the latter being replaced by Zeaxanthin in HL (Bonente *et al.*, 2011; Pinnola, Ghin, *et al.*, 2015). Also, LHCSR harbours lumen-exposed protonatable residues whose mutation (Ballottari *et al.*, 2016) or truncation (Liguori *et al.*, 2013) prevent quenching *in vivo*. Thus LHCSR, comprises both the functions, which are separated in PSBS: pH detection and catalysis of quenching (Bonente *et al.*, 2011; Peers *et al.*, 2009; Pinnola, Ghin, *et al.*, 2015).

The photophysical properties of LHCSR proteins have been studied on the pigment-complex reconstituted in vitro upon expression of the *C. reinhardtii* sequence in bacteria (Bonente *et al.*, 2011; Liguori *et al.*, 2013). The protein showed, when compared to other LHCBs, a shorter lifetime (<100ps vs 3.4 - 4.5ns) which was

further shortened at low pH (Bonente *et al.*, 2011), suggesting that quenching *in vivo* might require the establishment of interaction(s) with antenna proteins for excitation energy being spilled over to LHCSR in its quenched state. Alternatively, it was suggested that the on-off switch between a quenched and unquenched state is loose, already causing activation at low light intensities (Niyogi and Truong, 2013). A more "native" system was provided by the expression of LHCSR1 from *Physcomitrella patens* in tobacco and isolation of the tagged recombinant protein by affinity chromatography (Pinnola, Ghin, *et al.*, 2015). The major difference between the LHCSR proteins from *C. reinhardtii* and *P. patens* is the dependence on Zeaxanthin for the moss protein (Pinnola *et al.*, 2013) while the activity of LHCSR from *C. reinhardtii* is Zeaxanthin-independent. The role of Zeaxanthin in LHCSR appears to be, at least in part, allosteric since LHCSR dependence on de-epoxidation is very diverse among different species of unicellular algae (Quaas *et al.*, 2015) while the radical cation identified in both *C. reinhardtii* and *P. patens* has the spectroscopic features of Lutein (Bonente *et al.*, 2011; Pinnola *et al.*, 2016). Nevertheless, besides radical cation formation, an additional strong component, attributable to ET from Chl to Zeaxanthin was reported (Pinnola *et al.*, 2016). Thus, Lutein can replace Zeaxanthin in the $Car^{\circ+}$ dependent mechanisms (Li *et al.*, 2009; Pinnola *et al.*, 2016; Bonente *et al.*, 2011) while quenching by excitation energy transfer to carotenoids appears to require Zeaxanthin (Dall'Osto *et al.*, 2017; Pinnola *et al.*, 2016). It thus seems that the two mechanisms contributing to quenching in moss LHCSR are each localized in different antenna subunits in plants, namely $Car^{\circ+}$ in LHCB4 and excitation energy transfer to Zeaxanthin followed by a decay to the ground state in LHCII (Dall'Osto *et al.*, 2017), and that both are activated by PSBS.

The quenching process and contributions from Zea binding could be analysed in the PpLHCSR protein: the lifetime at neutral pH was 3.7ns, very similar to that of the major antenna LHCII, while the fully quenched protein exhibited an 80ps lifetime (Pinnola *et al.*, 2017). Intermediate conditions yielded a mix of ns vs ps lifetimes in different ratios, suggesting LHCSR can switch between two conformations with the extent of quenching being determined by the abundance of

the 80ps conformation. This was confirmed by single molecule spectroscopic studies (Kondo *et al.*, 2017).

It is interesting to speculate on the evolution of LHCSR and PSBS. LHCSR is found in all algal systems except red algae and glaucophytes and it likely arose from 2-helix forebears in Cyanobacteria. PSBS arose in the streptophyte line of green algae, which gave rise to liverworts and then to all land plants. It clearly evolved alongside (and possibly from LHCSR) and while LHCSR is present in green algae, streptophytes, liverworts, and mosses, but not in ferns, gymnosperms and flowering plants. Thus, in algae, energy quenching is largely accomplished by an LHCSR-dependent mechanism, while in land plants, from ferns on, PSBS is employed. Ferns and mosses, the first land colonizers, use PSBS and PSBS/LHCSR respectively, suggesting the transition from LHCSR to PSBS might be part of the adaptation from aquatic life to terrestrial life, or at least life in very shallow water (since PSBS is found in some green algae and certainly in streptophytes). Furthemore, LHCSR has been shown to be active when expressed in tobacco, implying that PSBS must have advantages over LHCSR, despite the fact that both are able to quench Chl fluorescence with similar efficiencies (as shown by the high level of quenching obtained in systems using each protein (Bonente *et al.*, 2008; Sello *et al.*, 2019)). Differences between the two systems include the site of interaction with core complex membranes which is LHCB5 in algae and LHCB4 in plants (Semchonok *et al.*, 2017; de Bianchi *et al.*, 2008) and the localization in the thylakoid membranes: the grana membranes for PSBS and stroma membranes for LHCSR (Pinnola, Cazzaniga, *et al.*, 2015; Girolomoni *et al.*, 2019). Thus the interaction of LHCSR3 with the core complex of PSII (Xue *et al.*, 2015) is restricted to PSII supercomplexes exposed to the interface with stroma membranes thus leaving a fraction of PSII units unprotected. Thus, it appears that the twofold process of quenching in PSBS (mentioned above) involving LHCB4 and PSBS to form the carotenoid cation ($Car^{\circ+}$) on one hand and LHCII and PSBS to form an ET from Chl to Zeaxanthin, followed by fast decay to the ground state (Dall'Osto *et al.*, 2017) is a strong evolutionary trait.

Conclusions

Although derived from photoprotective HLIPs proteins (Staleva *et al.*, 2015), LHCs evolved into an extremely diversified superfamily, with members devoted to harvest photons in specific narrow spectral bands such as LHCA4 to others which, at the opposite site of the functional range, do work in dissipating photon energy just absorbed by their interaction partners in the antenna system like LHCII. While photosynthetic reaction centres remained essentially unmodified through evolution, LHC proteins proved to be the critical and flexible factor in the evolution of photosynthetic organisms and their adaptation to the most diverse light regimes. It is clear that differences in the absorption range of LHCs are not only mediated by chromophore composition such as with FCP and LHCs, but are also driven by the protein environment. Besides light-harvesting, antenna complexes play essential roles in photoprotection. Since plants and algae generally seem to stay on the safe side and dissipate more energy than strictly necessary, improvements can be made to increase crop productivity for the production of food and fuel by engineering LHC proteins and/or their chromophores, which has been shown in tobacco (Kromdijk *et al.*, 2016). Two proteins have been shown to be essential for the activation of the photoprotection mechanisms, LHCSR and PSBS found in algae and plants respectively. The transition from LHCSR to PSBS upon land colonization, for Non-Photochemical Quenching, is a fascinating evolutionary development one that, together with the elucidation of the quenching mechanism, still requires investigation.

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Chapter 2

Functional analysis of LHCSR1, a protein catalyzing NPQ in mosses, by heterologous expression in *Arabidopsis thaliana*

A version of this chapter has been published in Photosynthesis Research:

Dikaios, I., Schiphorst, C., Dall'Osto, L., Alboresi, A., Bassi, R., & Pinnola, A. (2019). Functional analysis of LHCSR1, a protein catalyzing NPQ in mosses, by heterologous expression in Arabidopsis thaliana. *Photosynthesis Research*, 1–16. https://doi.org/10.1007/s11120-019-00656-3

Functional analysis of LHCSR1, a protein catalyzing NPQ in mosses, by heterologous expression in *Arabidopsis thaliana*

Ioannis Dikaios*[,1], Christo Schiphorst*[,1], Luca Dall'Osto[1], Alessandro Alboresi[1], Roberto Bassi[1] and Alberta Pinnola[1,2,3]

[1] *Department of Biotechnology, University of Verona, Verona, 37134, Italy*

[2] *Department of Biology and Biotechnology, University of Pavia, Pavia, 27100, Italy*

[3]corresponding author

[*]equal contribution

Abstract

Non-photochemical quenching, NPQ, of chlorophyll fluorescence regulates the heat dissipation of chlorophyll excited states and determines the efficiency of the oxygenic photosynthetic systems. NPQ is regulated by a pH sensing protein, responding to the chloroplast lumen acidification induced by excess light, coupled to an actuator, a chlorophyll/xanthophyll subunit where quenching reactions are catalyzed. In plants the sensor is PSBS while the two pigment-binding proteins Lhcb4 (also known as CP29) and LHCII are the actuators. In algae and mosses, stress-related light-harvesting proteins (LHCSR) comprise both functions of sensor and actuator within a single subunit. Here, we report on expressing the *lhcsr1* gene from the moss *Physcomitrella patens* into several *Arabidopsis thaliana npq4* mutants lacking the pH sensing PSBS protein essential for NPQ activity. The heterologous protein LHCSR1 accumulates in thylakoids of *A. thaliana* and NPQ activity can be partially restored. Transformation of double mutants lacking, besides PSBS, specific xanthophylls, allowed analyzing chromophore requirement for LHCSR-dependent quenching activity. We show that the partial recovery of

NPQ is mostly due to the lower levels of Zeaxanthin in *A. thaliana* in comparison to *P. patens*. Transformed *npq2npq4* mutants, lacking besides PSBS, Zeaxanthin Epoxidase, showed an NPQ recovery of up to 70% in comparison to *A. thaliana* wild type. Furthermore, we show that Lutein is not essential for the folding nor for the quenching activity of LHCSR1. In short, we've developed a system to study the function of LHCSR proteins by using heterologous expression in a variety of *A. thaliana* mutants.

Introduction

The need for a balance between light harvesting and photoprotection is one of the key driving forces that shaped adaptation of photosynthetic eukaryotic organisms on Earth (Genty et al. 1990; Müller et al. 2001; Baker 2008). Non-Photochemical Quenching (NPQ) of chlorophyll (Chl) fluorescence acts through modulating the dissipation of Chl excited states into heat and balances the efficiency of the photosynthetic systems *vs* the electron transport rate, thus avoiding photo-oxidative stress and photoinhibition due to excess light. NPQ includes components with different induction and relaxation kinetics: the fastest (1-2 minutes) and rapidly reversible type, qE, depends on a trans-thylakoid ΔpH promoted by excess light (Horton et al. 1996; Kramer et al. 1999; Kanazawa and Kramer 2002) which protonates specific residues on pH sensitive trigger proteins (Li et al. 2004; Ballottari et al. 2016); qZ, is activated in 8-10 minutes and also depends on low luminal pH through the activation of violaxanthin (Vio) de-epoxidase (VDE), a lumenal enzyme converting zeaxanthin (Zea) from pre-existing Vio. The slowest component, called qI, for photoInhibitory quenching, comprises components from the slow and reversible inactivation of Photosystem II (PSII) reaction centers as well as other long-term processes involved in acclimation to the light environment (Brooks et al. 2013). In some organisms, such as *Chlamydomonas reinhardtii* an additional component, qT, is due to the displacement of LHCII from PSII to PSI upon phosphorylation (Allorent et al. 2013). qE activation depends on a sensor for lumenal pH, induced by excess light, coupled to a Chl/xanthophyll actuator subunit where quenching reactions are catalyzed upon the establishment of specific

pigment-pigment interactions (Allorent et al. 2013). The protein PSBS is a typical pH sensor (Li et al. 2000) which does not bind chromophores (Dominici et al. 2002; Fan et al. 2015), but is able to activate quenching within the interacting antenna protein subunits Lhcb4 (CP29) (Ahn et al. 2008; de Bianchi et al. 2011) and LHCII (Ruban et al. 2007; Dall'Osto et al. 2017). In the case of algae the trigger of qE is LHCSR, a pigment binding protein (Peers et al. 2009) which also hosts protonatable residues (Liguori et al. 2013; Ballottari et al. 2016) thus comprising both sensing and catalytic functions in a single subunit (Bonente et al. 2011). The moss *Physcomitrella patens*, a descendant from an evolutionary intermediate between algae and plants, hosts both PSBS and LHCSR each active in qE (Alboresi et al. 2010; Gerotto et al. 2012) which suggests that LHCSR might be active in vascular plants. Modification of qE timescales in which PSBS and LHCSR1 are active, can improve crop productivity depending on the growth conditions (Horton 2000). Since the NPQ-activity of PSBS and LHCSR1 are cumulative in *P. patens* and LHCSR1 is not present in plants, re-introducing LHCSR1 in vascular plants could enhance the dynamic range of NPQ with positive effects on crop productivity. In this work we used the *npq4* mutant of *A. thaliana*, lacking PSBS and therefore qE, as a host for the expression of *P. patens* LHCSR1. We proceeded to verify the possibility of expressing LHCSR1 in vascular plants and its capability to complement the NPQ function in genotypes lacking PSBS. The availability of a large library of *A. thaliana* mutants affected in energy dissipation makes transformation by *Agrobacterium* mediated floral dipping (Clough and Bent 1998), an efficient tool for elucidation of the quenching-mechanism in LHCSR1. As a proof of concept, we determined the requirement of specific xanthophyll co-factors for LHCSR1-dependent quenching.

Material and Methods

Cloning of LHCSR1 cDNA, A. thaliana transformation and screening

The fragment corresponding to *LHCSR1* (Locus XM_024529130) was amplified from *P. patens* total cDNA obtained from 6 days old plants grown on minimal medium, RNA was isolated using TRI Reagent® Protocol (T9424, Sigma-Aldrich)

and cDNA was synthetized using M-MLV Reverse Transcriptase (M1302, Sigma-Aldrich) and Oligo(dT)$_{23}$ (O4387, Sigma-Aldrich). Primers including *attB* sequences for the gateway technology (Invitrogen™) were designed to anneal 27 base pairs upstream of the ATG codon (*Pp*LHCSR1attB1 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCCAATCTCGAGCTTTTGCT-3') and 107 base pairs downstream of the stop codon (*Pp*LHCSR1attB 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCGACTGCGAATCAATCAG AA-3'). The PCR-product was first cloned in pDONR™221 Vector (12536-017, Invitrogen™) and then recombined into the pH7WG2 binary vector (Karimi et al. 2002) to make the *35S::lhcsr1* construct. The accuracy of the cloning was verified by DNA digestion and sequencing and the plasmid was transferred to *Agrobacterium tumefaciens* strain GV3101 (Zhang et al. 2006). *A. thaliana* plants were transformed by the floral dip method and transgenic plants were selected on Moorashige-Skoog medium supplemented by hygromycin (25 mg L^{-1}) and carbenicillin (100 mg L^{-1}) (Clough and Bent 1998).

Plant material and growth conditions

P. patens protonema tissue was grown in petri dishes containing minimum $PPNO_3$ medium (Ashton et al. 1979) enriched with 0.5% glucose and solidified with 0.8% plant agar. Material was grown under controlled light and temperature conditions: 24°C, 16-h light/8-h dark photoperiod with a light intensity of 60µmol photons·m^{-2}·s^{-1}. *A. thaliana* plants (ecotype *Columbia*) were grown in controlled conditions of 8-h light/16-h dark with a light intensity of 100µmol photons·m^{-2}·s^{-1} under stable temperature (23^{o}C in light / 20^{o}C in dark).

Gel Electrophoresis

Total leaf extracts from transgenic *A. thaliana* plants were homogenized using plastic pestles in Laemmli buffer with 62.5 mM Tris pH 6.8, 10% glycerol, 5% SDS, 5% β-mercaptoethanol and loaded on a 15% (w/v) separating acrylamide gel (75:1 acrylamide/bis-acrylamide) with 6M Urea. After SDS-PAGE gel electrophoresis, proteins were transferred by western-blot on a polyvinylidene fluoride (PVDF) transfer membrane (Millipore) with the use of a Bio-rad blot

system and developed using specific LHCSR and CP43 or CP47 antibodies produced in the laboratory.

Thylakoid isolation and thylakoid fractionation

Thylakoids were purified from about 25-days old A. thaliana WT and transgenic plants (Berthold et al. 1981). Detached leaves from dark-adapted plants were harvested and homogenized in cold extraction buffer containing 0.02M Tricine-KOH pH 7.8, 0.4M NaCl, 0.002M $MgCl_2$, 0.5% milk powder, and protease inhibitors 5 mM ε-aminocaproic acid, 1 mM phenyl-methylsulfonyl fluoride and 1 mM benzamidine added right before the isolation. Homogenized leaves were then filtered, centrifuged at 1500g for 15 min at 4°C and then resuspended in a hypotonic buffer of 20 mM Tricine-KOH pH 7.8, 5 mM $MgCl_2$, 150 mM NaCl and the pre-mentioned concentrations of protease inhibitors. Resuspended thylakoids were centrifuged for 10min at 10,000g (4°C) followed by a second resuspension in a sorbitol buffer (10 mM HEPES-KOH pH 7.5, 0.4M Sorbitol, 15 mM NaCl and 5 mM $MgCl_2$). Thylakoid membranes were quantified and either used directly or stored in -80^{o}C.

Solubilization was performed as in (Morosinotto et al. 2010; Pinnola et al. 2015b, a). Isolated thylakoids were resuspended in 20 mM HEPES-KOH, pH 7.5, 15 mM NaCl, 5 mM $MgCl_2$ buffer at 1 mg Chl/ml and solubilized at 4 °C for 20 min in slow agitation with different amounts of α-DM ranging from 0.16 to 0.49% (w/v), always in the presence of 15 mM NaCl, 5 mM $MgCl_2$ and 10 mM HEPES-KOH, pH 7.5. Unsolubilized thylakoids were pelleted by centrifugation at 3.500g for 5 min. Partially solubilized grana membranes were instead pelleted with a further 30 min centrifugation at 40.000g. Solubilized complexes and small membrane patches remained in the supernatant. Membrane pellet was washed with 15 mM NaCl, 5 mM $MgCl_2$ and 20 mM HEPES-KOH, pH 7.5, centrifuged for 30 min at 30.000g and finally resuspended in 0.4 M Sorbitol, 10 mM HEPES-KOH, pH 7.5, 15 mM NaCl, 5 mM $MgCl_2$ frozen in liquid nitrogen and stored at –80°C until use.

Pigment-protein complexes separation with Deriphat-PAGE

Non-denaturating Deriphat-PAGE was performed as previously described (Peter et al. 1991) with some modifications: stacking gel of 3.5% (w/v) acrylamide (38:2

acrylamide/bis-acrylamide) and separating acrylamide gel was prepared at different fixed or gradient concentration depending on the purposes. Acrylamide concentrations are specified along the text. Thylakoids from wild type and transgenic plants corresponding to a final Chl concentration of 0.5mg were washed with 5mM EDTA and then resuspended in 10mM HEPES pH 7.5. Samples were then solubilized with 0.8% n-*Dodecyl* α-D-*maltoside* and 10 mM HEPES pH 7.5 by vortexing thoroughly for 1min. Solubilized samples were kept 10 min in ice and then centrifuged at 15,000g for 10min to pellet any insolubilized material and then loaded.

Fluorescence measurements

In vivo Chl fluorescence was measured at room temperature after leaves were dark adapted for 45min, by FC 800MF closed FluorCam Video-imaging system (Photon Systems Instruments, Czech Rep.) and PAM-100 (Walz, Germany) fluorometers. For every measurement a saturating pulse of 4000μmol photons·m^{-2}·s^{-1} and actinic light with an intensity of 1200μmol photons·m^{-2}·s^{-1} were applied. Fv/Fm and NPQ parameters were calculated as (Fm−Fo)/Fm and (Fm−Fm′)/Fm′ respectively.

Pigment composition analysis (HPLC)

A. thaliana leaves or *P. patens* protonema tissue was frozen in liquid nitrogen and homogenized using plastic pestles. Pigments were extracted in 80% ice-cold acetone (buffered with $NaHCO_3$) and analyzed by High-Pressure Liquid Chromatography (HPLC) after a twostep centrifugation at 21000g for 10 min at 4°C.

9-aminoacridin measurements

Intact chloroplasts were isolated from 4- to 5- weak old *A. thaliana* (*npq4+LHCSR1*) plants or 6 days old *P. patens psbs-lhcsr2 ko* (PzL_2) based on the method from (Munekage et al. 2002). Tissue was homogenized using a potter in ice-cold buffer containing 330mM sorbitol, 20mM Tricine/NaOH (pH 7.6), 5mM EDTA, 5mM EGTA, 10mM $NaHCO_3$, 5mM $MgCl_2$, 0.1% (w/v) BSA and 1.87mM Sodium L-ascorbate. The homogenized tissue was filtered through a nylon mesh and the filtrate was centrifuged at 2000g for 5min in a pre-chilled centrifuge (4 °C).

The pellet was resuspended in an ice-cold buffer containing 300mM Sorbitol, 10mM HEPES/NaOH (pH 7.6), 5mM $MgCl_2$, 10mM $NaHCO_3$, 2.5mM EDTA and 1.87mM of Sodium L-ascorbate. Samples were kept on ice until right before the measurements. Chloroplasts were diluted to 25µg/mL Chl in a buffer at room temperature containing 50mM Tricine/NaOH (pH 8.0), 100mM NaCl, 10mM $MgCl_2$ and 9-aminoacridine (2µM) and measurements were performed at different light intensities 50, 200, 500 and 800µmol photons·m^{-2}·s^{-1}. Fluorescence measurements were recorded on a Fluoromax-3 (Horiba scientific), excitation wavelength 400nm, emission measured at 430nm. Electron transport was induced by using the actinic light of a pulse-amplitude modulated fluorimeter (Heinz-Walz) equipped with a red filter (600-750nm), 40 seconds of dark adaptation, 80 seconds illumination and 40 seconds of recovery in the dark.

Results

1.1 LHCSR1 expression in *A. thaliana npq4* mutant

The coding sequence of LHCSR1 was amplified from cDNA synthetized from *P. patens* protonema, cloned in pH7WG2 vector under the control of the constitutive 35S promoter and used for *Agrobacterium*-mediated transformation of *npq4* mutant plants. The *npq4* mutants are devoid of qE due to the absence of PSBS. Transgenic seeds were collected and grown on hygromycin-B, resistant seedlings were transferred to soil, together with *A. thaliana* wild type (WT) and *npq4* control plants. Leaf extracts from *A. thaliana* genotypes and *P. patens* protonema tissue were analyzed by western blotting using α-LHCSR (Pinnola et al. 2013) and α-CP43 antibodies. WT and *npq4* plants showed no reaction with α-LHCSR while CP43 was detected in all samples (Fig. S1b). In *P. patens* both LHCSR1 and LHCSR2 were detected. In *A. thaliana* a single band, corresponding to LHCSR1, was obtained in hygromycin-resistant *A. thaliana* plants with a mobility matching the native LHCSR1 protein in *P. patens* thylakoid membranes. This suggests that *P. patens* LHCSR1 is both expressed and processed to its mature form in *A. thaliana*. The strongest LHCSR1 expressors among the transformed *A. thaliana npq4* lines were selected (C1, C3 and A5) and used to create homozygous lines,

these lines contained multiple insertions which was established from the segregation pattern in later generations.

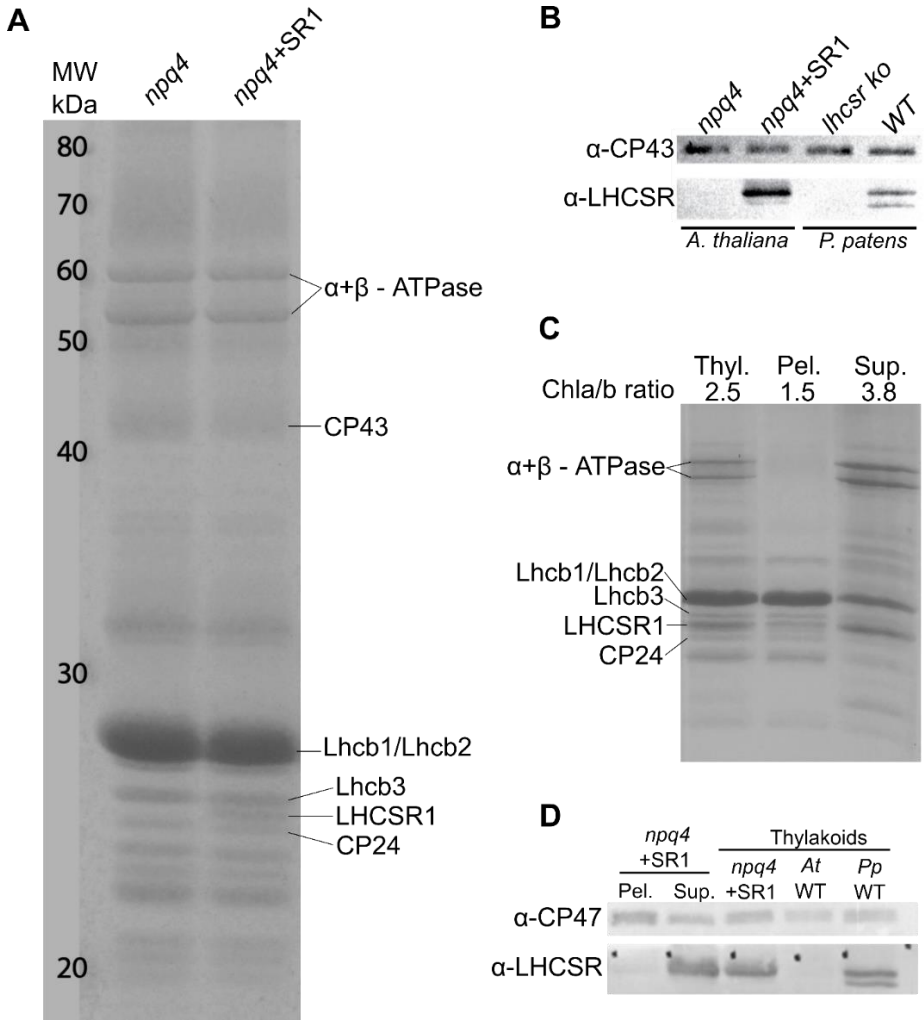

Figure 1 *A. thaliana thylakoid membrane fractionation and analysis of LHCSR distribution in the individual fractions.* ***a*** *Coomassie-stained SDS-PAGE separation of thylakoid proteins isolated from npq4 plants and npq4 plants expressing LHCSR1 (npq4+SR1). LHCSR1 and other bands are indicated on the right side of the gel.* ***b*** *Western blot analysis of thylakoid proteins isolated from A. thaliana npq4 plants and npq4 plants expressing LHCSR. As control, thylakoids from P. patens WT and lhcsr KO were loaded.* ***c*** *Coomassie-stained SDS-PAGE of fractionated thylakoid membranes from A. thaliana npq4+LHCSR1 using 0.47% α-DM. Thylakoid membranes (Thyl.), pellet enriched in grana fractions (Pel.) and the supernatant enriched in stroma membranes (Sup.). Gels were loaded on Chl basis, 4 µg for thylakoids and 2.7µg for both the pellet and supernatant fractions. The Chl a/b ratio is indicated above the gel.* ***d*** *western blot analysis of the fractionated thylakoid membranes and thylakoids from A. thaliana npq4, npq4+LHCSR1, WT and P. patens WT as controls*

It was verified that the T4 generation of line C1 was stable and further experiments, unless otherwise indicated, were performed on the T5 generation of this line. A quantitative western blot showed that these plants contain 82.8±1.8% of LHCSR1 in comparison to the LHCSR1-only *P. patens psbs-lhcsr2 knock-out (ko)* (Fig. S2).

1.2 LHCSR1 localization in *A. thaliana* thylakoid membranes

Thylakoid membranes from *A. thaliana npq4* plants expressing LHCSR1 and control *npq4* plants were purified and analyzed by SDS-PAGE (Fig. 1a). A band with the apparent molecular weight of LHCSR1 was present with a mobility between Lhcb3 and Lhcb6 (CP24) in the transformed plants but not in the background line *npq4*. The new band from the LHCSR1 expressing *A. thaliana* thylakoids was excised from gel and submitted to mass spectrometric analysis which yielded 10 peptides covering 58% of the mature protein sequence (Fig. S3). Each peptide matched the theoretical mass calculated from the DNA sequence, implying no post-translational modifications were present within the identified fragments. No other identifiable changes in protein composition could be detected between the two genotypes (Fig. 1a). Furthermore, western blot analysis confirmed that the α-LHCSR antibody reacted against the LHCSR1 protein accumulated in the thylakoid membranes of the transformed plants (Fig. 1b) with the same electrophoretic mobility as the native protein from *P. patens.* LHCSR1 and LHCSR2 were detected in the WT *P. patens* thylakoids, but not in the *P. patens lhcsr1-lhcsr2 ko* thylakoid membranes (Fig. 1b).

The distribution of LHCSR1 in the thylakoid domains of *A. thaliana*, was assessed by thylakoid fractionation with n-dodecyl α-D-maltoside (α-DM) (Morosinotto et al. 2010; Pinnola et al. 2015b, a). The procedure yielded a pellet enriched in grana membranes and a supernatant comprising the stroma lamellae. The *Coomassie*-stained SDS-PAGE gel confirmed PSI and ATPase were in the stroma-derived supernatant fraction while the PSII core subunit as well as LHCII and Lhcb6 (CP24) were enriched in the pellet, i.e. the grana partitions (Fig. 1c). A band with the apparent molecular weight corresponding to LHCSR1 was highly enriched in the supernatant, suggesting that recombinant LHCSR1 was localized in thylakoid stroma-exposed membranes of transformed *A. thaliana npq4*. This was confirmed

by western blot (Fig. 1d) and step-solubilization with increasing concentrations of α-DM detergent, namely 0.16, 0.24, 0.32, 0.39 and 0.47% α-DM (Fig. S4). Immunoblotting showed that LHCSR1 was already enriched in the stromal fraction at 0.16% α-DM with a Chl *a*/*b* ratio >6.0 and a polypeptide composition, including PSI and ATPase typical of stroma membranes (Fig. S4a, b). Low amounts of LHCSR1 were found in the pellet fractions up to 0.32% α-DM suggesting that the protein might also be localized in the margins of *A. thaliana* thylakoids.

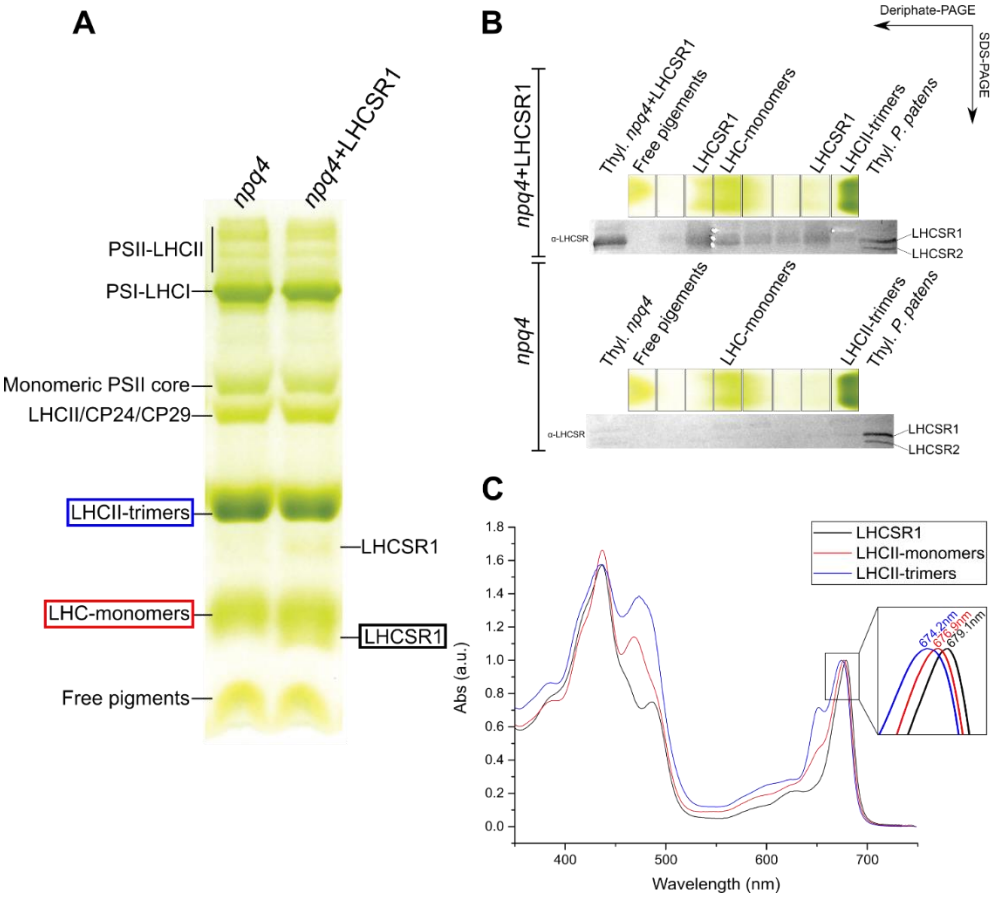

Figure 2 *Deriphat-PAGE analysis of A. thaliana thylakoid membrane protein complexes. a Thylakoid membranes (30 µg of Chl) of npq4 and npq4+LHCSR1 plants solubilized with 0.8 % (w/v) α-DM were subjected to Deriphat-PAGE. PSII and PSI complexes, together with various LHCs are indicated on the left side of the gel. Complexes more abundant in npq4+LHCSR1 than in npq4 plants are labelled as LHCSR1 on the right side of the gel. Coloured rectangles correspond to the bands used for the absorption spectra analysis in (panel c). b Deriphat-PAGE (7%) of unstacked thylakoids from A. thaliana WT and npq4+LHCSR1, solubilized in 0.8% α-DM. Bands were eluted in 10mM HEPES/0.03% α-DM. Eluted fractions were loaded on SDS-PAGE and immunoblotted against α-LHCSR. c Absorption spectra of the bands taken from the Deriphat-PAGE (panel a), LHCSR1, LHCII-monomers and LHCII-trimers of A. thaliana npq4+LHCSR1*

1.3 Pigment-binding to recombinant LHCSR1 and their spectra

LHCSR is a pigment-binding protein in *C. reinhardtii* and *P. patens* (Bonente et al. 2011; Pinnola et al. 2013). To verify that recombinant LHCSR1 expressed in *A. thaliana* did actually refold properly with pigments, thylakoid membranes were analyzed by Deriphat PAGE (Fig. 2a). Although the protein composition was similar between the two genotypes, *A. thaliana npq4*+LHCSR1 did contain two additional bands with respect to *A. thaliana npq4*, migrating respectively just below the monomeric LHC band and in between LHC monomers and trimers. Gel slices were excised from the gel and further separated by denaturing SDS-PAGE, followed by western blotting. The two "additional" bands in the gel from *A. thaliana npq4*+LHCSR1 showed strong reaction towards the α-LHCSR antibody (Fig. 2b). Fainter reactions were also obtained with fractions from in between the two bands but not with those at lower or higher mobility, suggesting that LHCSR1 migrated initially as a dimeric Chl binding protein which partially dissociated into monomers during solubilization and/or electrophoretic migration.

LHCSR proteins have a characteristically red-shifted absorption spectrum with respect to other LHC proteins (Bonente et al. 2011; Pinnola et al. 2013, 2015b). Absorption spectra recorded from extracted gel bands showed a red-shifted Q_y peak at 679.1 nm with respect to LHCII trimers (674.2 nm) and LHC monomers (676.9 nm), typical for LHCSR1 (Bonente et al. 2011; Pinnola et al. 2017) (Fig. 2c). Also, the LHCSR1-containing band was depleted of Chl *b* with respect to the bands from other LHCs, thus copying the properties of recombinant LHCSR proteins either refolded *in vitro* or expressed in tobacco (Bonente et al. 2011; Pinnola et al. 2015b).

2.1 NPQ activity of LHCSR1 in *A. thaliana*

As previously mentioned, the NPQ activity of PSBS and LHCSR is additive and independent in *P. patens* plants (Alboresi et al. 2010; Gerotto et al. 2012). It was tested whether LHCSR1 could confer a light dependent *in vivo* quenching activity in the *A. thaliana npq4* mutant. Therefore, the Chl fluorescence quenching of *A. thaliana* WT, *npq4* plants and the transformed lines were measured using Chl fluorescence imaging. The protocol consisted of a 45min dark adaptation of the leaves, followed by 5min white actinic light (1200µmol photons·m^{-2}·s^{-1}*)* and 5min of dark recovery (Fig. 3).

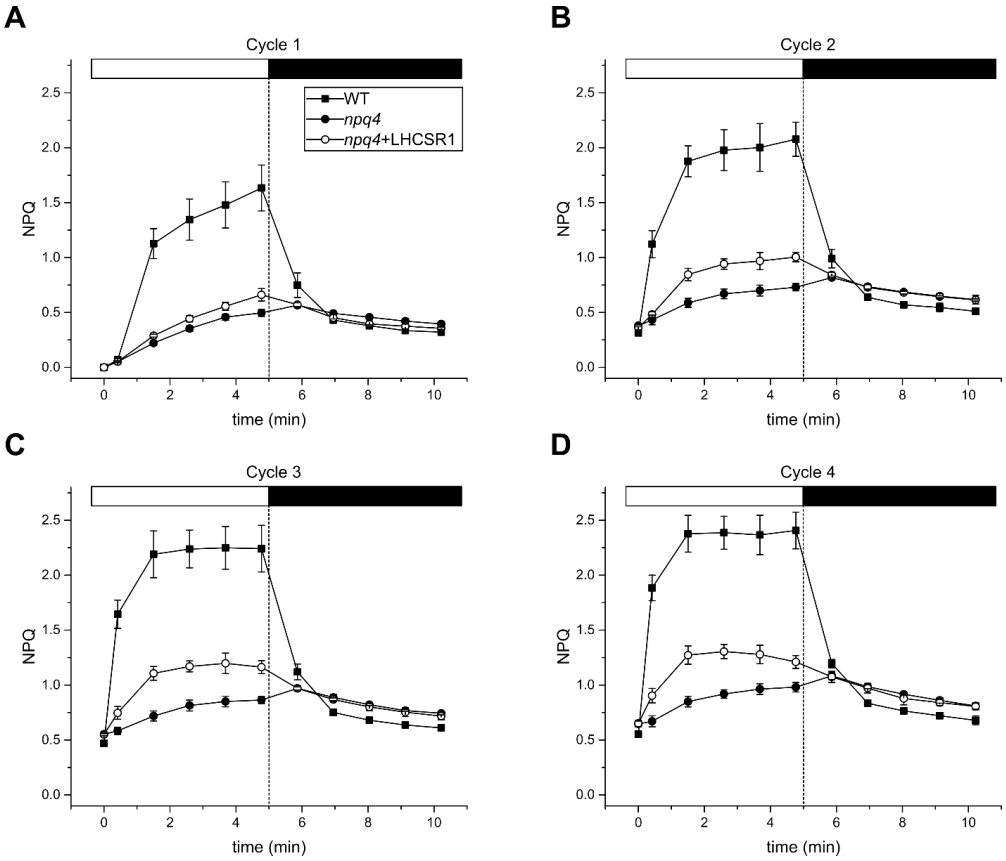

Figure 3 *NPQ kinetics (n=4) in A. thaliana WT, npq4 and npq4+LHCSR1. WT (Black squares), npq4 (black circles) and npq4+LHCSR1 (open circles). Plants were dark adapted for 45 minutes before the measurement, 4 cycles of 5min actinic light (1200µmol photons·m^{-2}·s^{-1}) and 5min dark, as described in the M&M. The four cycles are depicted by a-d respectively.*

When the protocol was applied to dark-adapted leaves, only a small difference in quenching activity was observed, suggesting the expression of LHCSR1 did not confer significant NPQ activity (Fig. 3a). However, when the same protocol was applied for the second-time, larger differences between *npq4* and *npq4*+LHCSR1 were observed (Fig. 3b). Two additional cycles of NPQ induction and relaxation were applied, where the differences were even further pronounced between the second and third measurement, the NPQ was very similar between the third and the fourth measurement (Fig. 3c, d). Interesting to note is that the *npq4* mutant showed a characteristic transient increase of quenching at the first point in the dark after switching off the actinic light, this jump was not detected in plants containing PSBS nor was it detected in lines expressing LHCSR1 (Fig. 3a-d). Since the accuracy of these NPQ measurements depend on the accuracy of the Fm measurement. All the

Fv/Fm values of the different NPQ measurements were included in Table S1 and S2. Fv/Fm values were found to be very similar for all the different LHCSR1 expressing lines (i.e. below 2%).

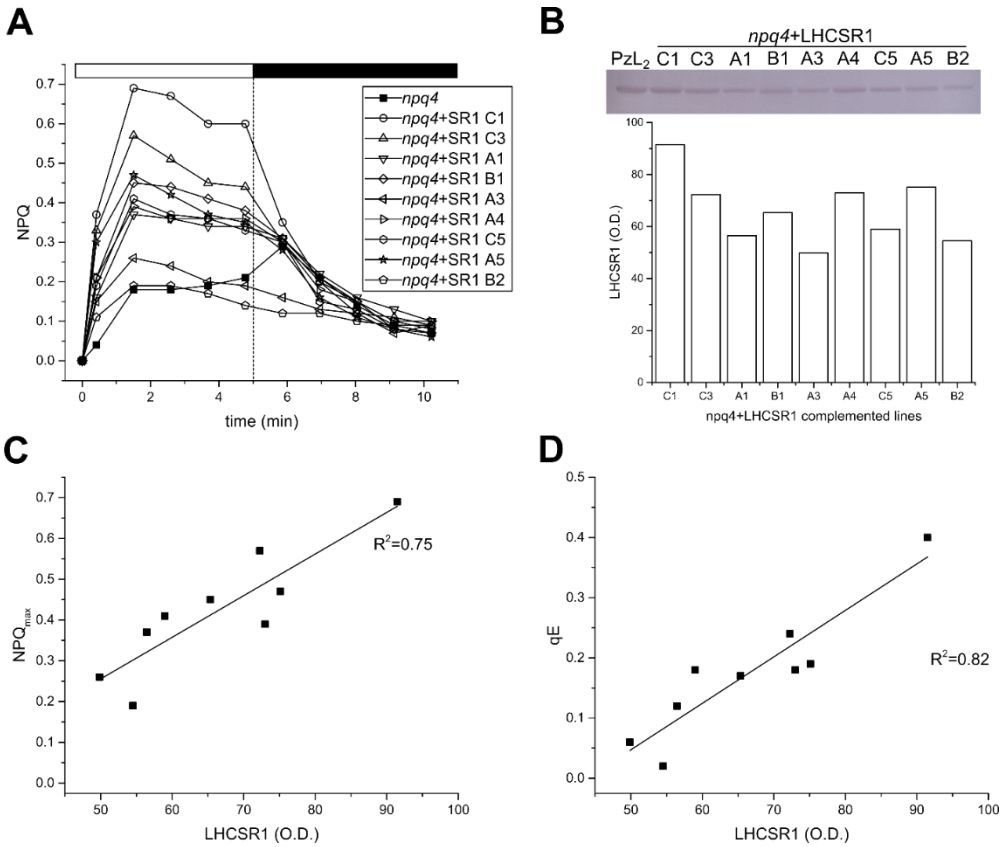

Figure 4 *Correlation between NPQ activity and LHCSR1 accumulation. NPQ measurements of the T2 generation of the npq4+LHCSR1 lines (n=9).* ***a.*** *Leaves were dark adapted for 45min, pre-treated with 1200µmol photons·m^{-2}·s^{-1} of actinic light for 15min and left to relax in the dark for 10min before the NPQ measurement* ***b*** *After the NPQ measurement total leaf extracts from each line were loaded on an SDS-PAGE on a basis of 0.75µg Chl and immuno-blotted against α-LHCSR antibodies. Thylakoids from P. patens psbs-lhcsr2 ko (PzL$_2$) were loaded as a control. The O.D. of LHCSR1 was determined.* ***c*** *Protein level plotted with the maximum NPQ, yielding a positive correlation of R^2=0.75.* ***d*** *Correlation between qE and the protein level (R^2=0.82). qE recovery is calculated as the NPQ of the last point in the light phase minus the second point in the dark phase*

2.2 LHCSR1 and zeaxanthin synthesis

Since Zea has a major influence on the quenching activity of LHCSR1 (Pinnola et al. 2013), the slow onset of LHCSR1-dependent NPQ activity in *A. thaliana* suggests that Zea accumulation might be limiting. Therefore, the leaf pigment content was determined by HPLC analysis of the *npq4* and two independent *npq4*+LHCSR1 lines during two cycles of 10min illumination followed by a 10min dark relaxation. Zea accumulated to the same level in both genotypes (Table S3) at the end of each dark or light phase while the 10-minute dark periods did not allow for a decrease in Zea level. We conclude that the repeated cycles of illumination (Fig. 3) lead to an increased NPQ activity, which is consistent with the accumulation of Zea (Table S3).

2.3 Correlation between LHCSR1 accumulation level and NPQ activity

To verify whether NPQ activity correlated with the amount of LHCSR1, 9 lines with different levels of NPQ were selected (Fig. 4a). Total leaf extracts were titrated with an α-LHCSR polyclonal antibody (Fig. 4b). Both qE and total NPQ activities linearly correlated with the level of LHCSR1. An estimation of the qE was determined by differences between NPQ values recorded at the end of the 5 min light period and upon 2 minutes of dark relaxation, allowing for a rapid estimation of qE activity (Dall'Osto et al. 2014). The NPQ activity per LHCSR1 unit was lower in *A. thaliana* with respect to *P. patens* since LHCSR1-only mosses did show an NPQ score 3-fold higher than the transformed *A. thaliana npq4* lines (Fig. 5a), while the level of LHCSR1 in *P. patens psbs-lhcsr2 ko* was only 1.2-fold higher (Fig. S2).

2.4 LHCSR1-dependent NPQ in *A. thaliana*: dependence on light intensity

In order to investigate the reasons for the low LHCSR1 activity in *A. thaliana vs. P. patens*, we verified the hypothesis that mosses might differ from *A. thaliana* for their light intensity dependence of LHCSR1 activity due to adaptation to shaded habitats of mosses. To this aim, three *npq4*+LHCSR1 lines with high and intermediate NPQ activity at 800µmol photons·m^{-2}·s^{-1} were selected and measured at a series of actinic light intensities: low light intensity (100µmol photons·m^{-2}·s^{-1}) up to 1000µmol photons·m^{-2}·s^{-1}. Before each measurement, leaves were dark-adapted for 45min, pre-treated with actinic light (800µmol photons·m^{-2}·s^{-1}) for

15min in order to accumulate equal Zea levels and left to relax for 10min in the dark. Leaves from *A. thaliana* WT and *npq4* plants of the same age were used as controls. At the lowest light intensity, transient NPQ was observed in all *A. thaliana* genotypes, which rapidly dropped, likely due to activation of the ATPase dissipating the ΔpH for ATP synthesis (Fig. 6). However, as the intensity of actinic light increased, plants activated NPQ and the LHCSR1 transformed lines already showed activity at 200µmol photons·m^{-2}·s^{-1}. Peak activity was reached after 2-3 minutes light exposure and the NPQ level was maximal at 400 and 600 µmol photons·m^{-2}·s^{-1}, with lower values at both lower and higher actinic light intensities. WT *A. thaliana*, besides showing at least twofold higher NPQ values, also did show strikingly different NPQ kinetics, monotonously rising under actinic light conditions and only relaxing when light was switched off. The partial relaxation of NPQ under actinic light could be explained by a relaxation of lumen acidity after 3 minutes of light treatment.

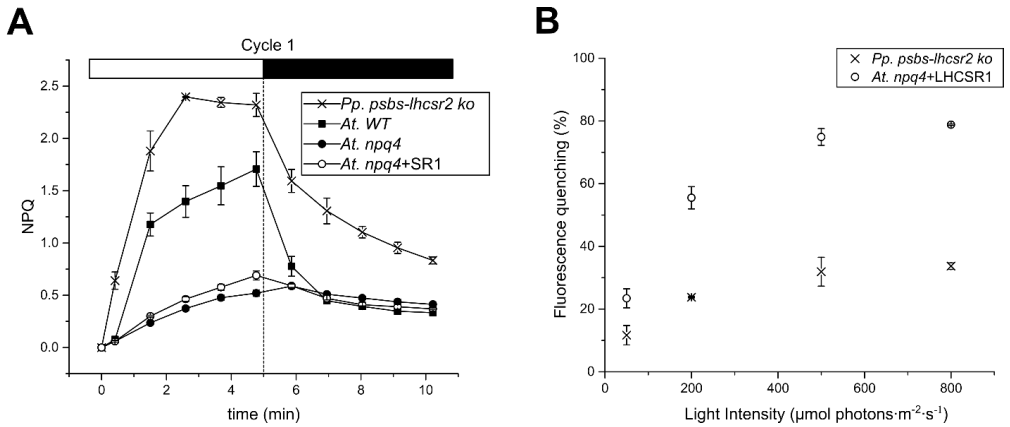

Figure 5 *Comparison of ΔpH and NPQ between P. patens psbs-lhcsr2 ko and A. thaliana.* ***a*** *Comparison of NPQ (n=4) between A. thaliana WT, npq4, npq4+LHCSR1 (npq4+SR1) and P. patens psbs-lhcsr2 ko. Fourth cycle of NPQ measurements at 1200µmol photons·m^{-2}·s^{-1} (5min light and 5min dark relaxation).* ***b*** *9-aminoacridin measurements (n=3) in isolated chloroplasts of A. thaliana npq4+LHCSR1 and P. patens psbs-lhcsr2 ko at different light intensities (50, 200, 500 and 800µmol photons·m^{-2}·s^{-1} of red light)*

In order to verify whether the activation of LHCSR1 might be affected by the amplitude of the pH gradient formation, we proceeded to measure the ΔpH through

thylakoid membranes at different light intensities. To this aim, chloroplasts of *A. thaliana npq4*+LHCSR1 plants and *P. patens psbs-lhcsr2 ko* were illuminated in the presence of the fluorophore 9-aminoacridine (9-AA). 9-AA fluorescence is quenched upon protonation when the chemical diffuses through the thylakoids into the lumen dependent on the trans-membrane pH gradient. Fig. 5b shows the 9-AA fluorescence quenching in *A. thaliana* vs *P. patens* at different light intensities, implying a different capacity of building up a trans-thylakoid pH gradient between the two organisms. Despite the fact that *A. thaliana* was able to reach higher ΔpH levels than *P. patens*, the NPQ activity of LHCSR1 in *A. thaliana* was lower with respect to *P. patens* (Fig. 5a).

3.1 LHCSR1 expression and NPQ activity in xanthophyll biosynthesis mutants

The above results imply that LHCSR1 proteins expressed in *A. thaliana npq4* can partially complement the lack of PSBS. When purified from *Physcomitrella patens*, LHCSR1 binds lutein (Lut) and Vio, part of which are substituted by Zea upon high light treatment (Pinnola et al. 2013). To identify the role of these xanthophylls for activation of LHCSR1 in *A. thaliana* we proceeded with the transformation of the *lhcsr1*-gene in the following *A. thaliana* double mutants: (*i*) *npq1npq4*, unable to accumulate Zea due to the lack of violaxanthin de-epoxidase (*vde*); (ii) *npq2npq4*, a mutant accumulating Zea due to the absence of zeaxanthin epoxidase (*zep*) (*iii*) *lut2npq4*, the lutein-less genotype defective in the lycopene ε-cyclase activity, which compensates missing Lut with increased levels of Vio.

Transformation of the *npq1npq4* mutant with LHCSR1 and selection in hygromycin yielded 16 stable lines, which accumulated LHCSR1 as assessed by western blot analysis (Fig. S5b), implying LHCSR1 can be expressed and accumulated in the absence of Zea (Fig. S5b). No major differences in the size or shape of the transformed plants were detected (Fig. S5a). The NPQ activity of *npq1npq4* plants and 3 independent transformed lines was measured by video-imaging following the initial protocol (see M&M). The quenching activity of the transformed lines was the same as in the *npq1npq4* background and did not increase during the subsequent cycles of illumination, failing to reveal any difference between *npq1npq4* (i.e. control) and the transformed *npq1npq4*+LHCSR1 plants

(Fig. 7a, b; Fig. S6a-d). This result is in agreement with previous reports in the homologous system *P. patens* showing that LHCSR1 requires Zea for quenching (Pinnola et al. 2013).

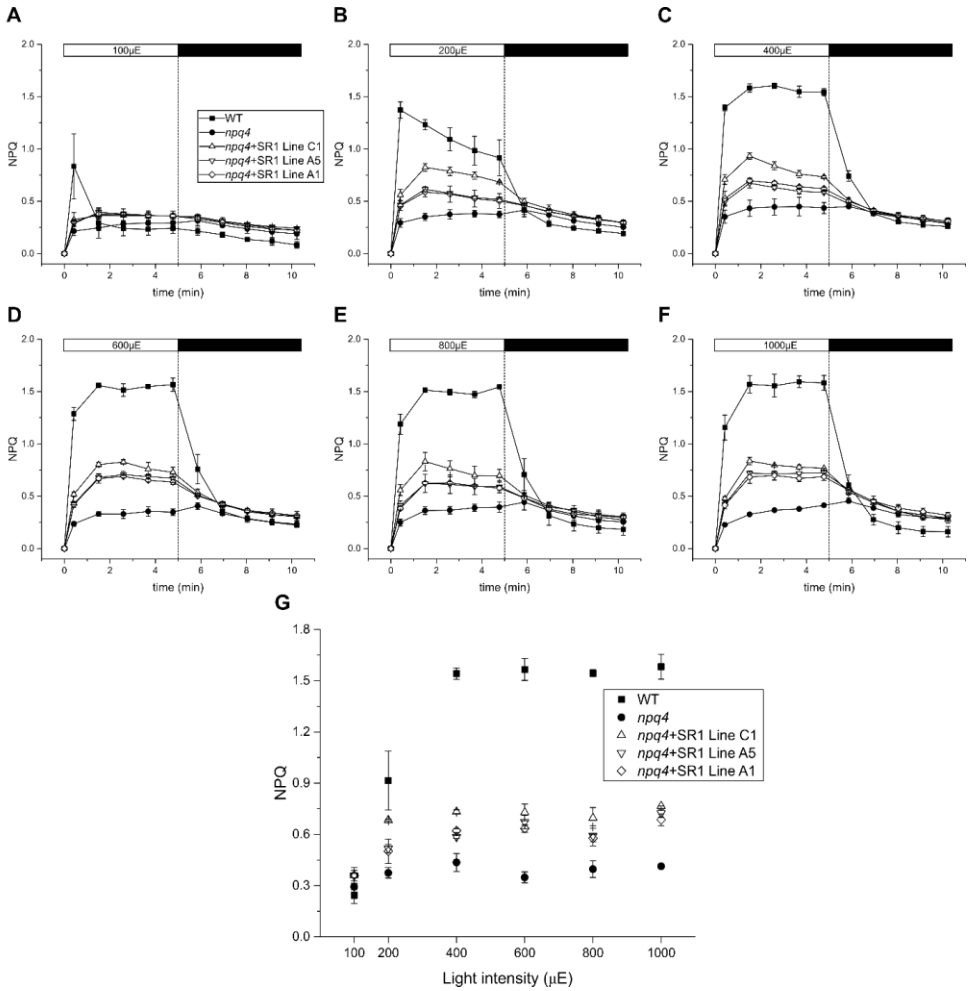

Figure 6 *NPQ activity of npq4+LHCSR1 lines in various light intensities. Three different A. thaliana npq4+LHCSR1 lines with high and intermediate NPQ activation (line C1, A1 and A5) were tested in a variety of actinic light intensities. Leaves (n=3) were dark adapted for 45min, pre-treated with 800µmol photons·m^{-2}·s^{-1} of actinic light for 15min and left to relax in the dark for 10min before the NPQ measurement. Each measurement corresponds to one single NPQ cycle of 5min different with different actinic light intensities and 5min dark recovery. The actinic light intensities used were: 100, 200, 400, 600, 800 and 1000µmol photons·m^{-2}·s^{-1} (µE) from* ***a-f*** *respectively. Leaves from A. thaliana WT and npq4 were used as controls.* ***g*** *NPQ of the last point in the light plotted against the different light intensities.*

The *npq2* mutant lacks Vio and accumulates full levels of Zea as well as Lut (Niyogi et al. 1998; Peers et al. 2009). Transformation of the *npq2npq4* mutant

yielded three plants accumulating LHCSR1 (Fig. S7). NPQ activity differed with respect to *npq4*+LHCSR1 in that it appeared already during the first cycle of illumination in dark-adapted plants (Fig. 7c, d; Fig. S8a-d). Furthermore, the total amount of NPQ in these plants was much higher, reaching up to 70% of *A. thaliana* WT. These observations indicate that the build-up and level of Zea are one of the limiting factors in the NPQ activity of LHCSR1. During the fourth cycle the maximal NPQ was reached after 2 minutes and showed the same partial relaxation kinetics as the *npq4*+LHCSR1. This relaxation, however, could already be observed during the third cycle in the *npq2npq4* transformed lines, but not in *npq4*+LHCSR1.

LHCSR1 expression in the *lut2npq4* yielded no major phenotypic differences between control and LHCSR1–transformed *lut2npq4* plants (Fig. S9a). Total leaf extracts from the transformed lines were analyzed by western blot with the α-LHCSR antibodies (Fig. S9b), showing that the protein was processed to its mature form without Lut. Using the 4-cycle actinic light protocol, the control and transformed lines were essentially indistinguishable in the first cycle (Fig. 7e). However, upon the second cycle the curves of the different genotypes became more shifted towards higher values with the exception of *lut2npq4*, which remained unchanged (Fig. S10). Two additional cycles further increased the difference in NPQ between *lut2npq4* and the transformed lines (Fig. 7f). Two features characterized these measurements with respect to the *npq4*+LHCSR1 genotype: first, the higher level of qE obtained in the *lut2npq4*+LHCSR1 with respect to *npq4*+LHCSR1; second, that the maximal NPQ values were obtained at the end of the illumination (5 min) rather than at the second minute as previously observed with the *npq4*+LHCSR1 and *npq2npq4*+LHCSR1 genotypes, consistent with a delay in reaching full de-epoxidation of Vio bound to slowly exchanging binding sites (Morosinotto et al. 2002).

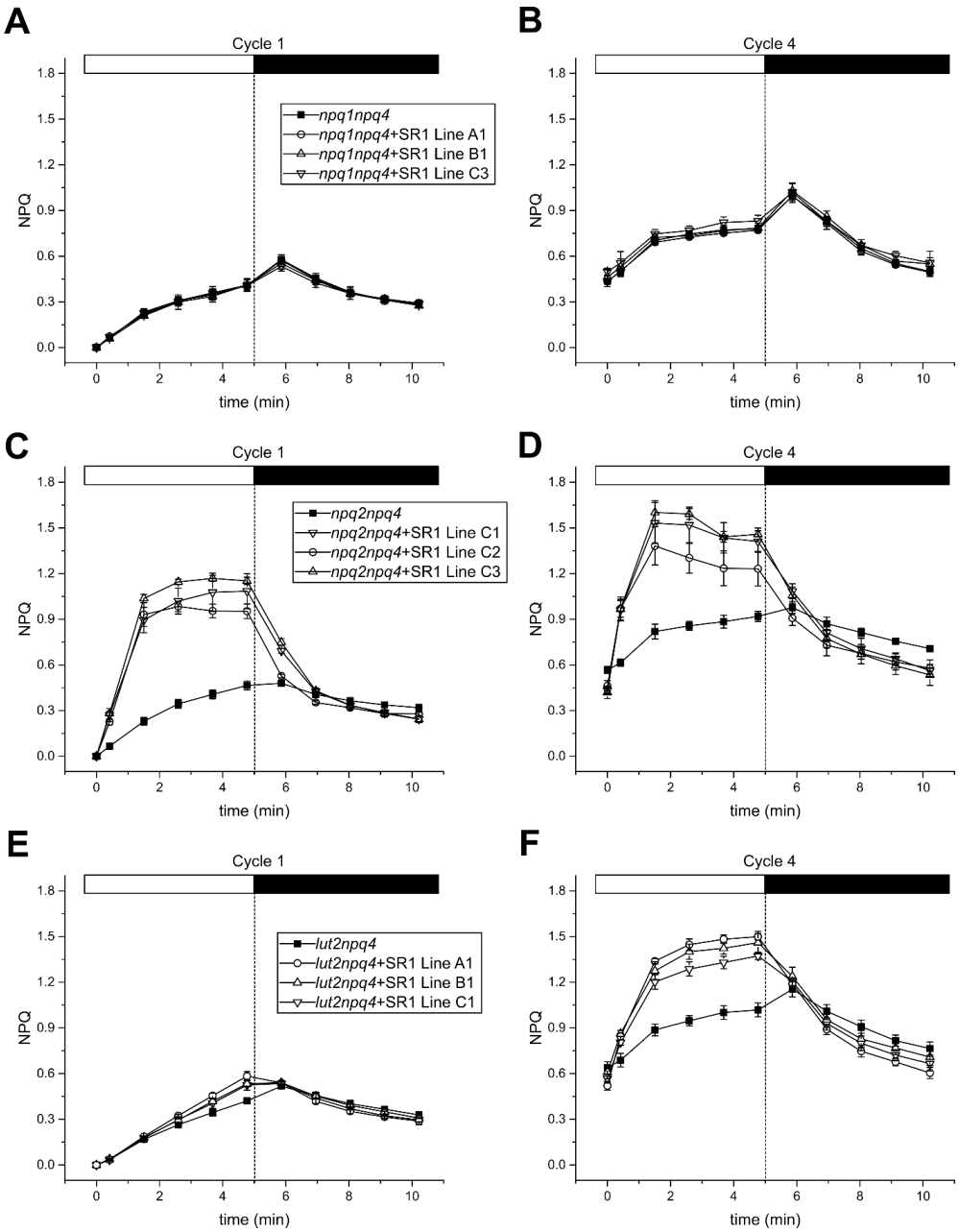

Figure 7 *NPQ measurements in A. thaliana mutants, lacking specific xanthophyll's, transformed with LHCSR1. Four successive NPQ cycles were measured (n=3) as described in M&M. The first and fourth cycle are shown for each transformed mutant.* ***a, b*** *first and fourth NPQ measurement in npq1np4 and three independent npq1npq4 lines transformed with LHCSR1 (npq1npq4+SR1).* ***c, d*** *first and fourth NPQ measurement in npq2npq4 and three npq2npq4 lines transformed with LHCSR1 (npq2npq4+SR1).* ***e, f*** *first and fourth NPQ measurement in lut2npq4 and three lut2npq4 lines transformed with LHCSR1 (lut2npq4+SR1).*

Discussion

While expression in tobacco has proven instrumental for purification of the LHCSR1 protein for biochemical and structural studies (Pinnola et al. 2015b), *A. thaliana* is a choice for functional studies due to the availability of a large collection of mutants and the easiness of transformation procedures. Here we show that the moss LHCSR1 protein can be expressed in *A. thaliana* to form a pigment-protein complex indistinguishable from the holoprotein purified from *P. patens* as judged from the mass spectrometric analysis and visible absorption spectra (Fig. 2c, S3). Also consistent with previous reports, LHCSR1 was found both as a monomer and dimer in non-denaturing green gels, suggesting its aggregation state in thylakoids is a dimer which, in part, monomerizes during solubilization and fractionation (Pinnola et al. 2015b). LHCSR1 purified from *P. patens*, binds Chl *a*, Lut and Vio with sub-stoichiometric levels of Chl *b*, where Vio is largely substituted by Zea during high light treatment (Pinnola et al. 2013, 2015b). Here we show that LHCSR1 from *P. patens* can be expressed in *A. thaliana npq4* mutants at levels comparable to that found in moss. Due to the absence of PSBS, the host lines did not show NPQ activity upon high light treatment (Li et al. 2000). Thus NPQ detected in transformed plants can be attributed to LHCSR1 based on the following observations: (i) quenching was only observed in LHCSR-transformed genotypes (Fig. 3); (ii) quenching was proportional to the level of LHCSR1 accumulation (Fig. 4); (iii) no LHCSR1-dependent quenching activity was observed in the *npq1npq4* background, lacking Zea (Fig. 7a, b), in agreement with the observation that the *vde* ko mutant in *P. patens* lost 95% of quenching activity (Pinnola et al. 2013); (iv) higher and fast-developing quenching was observed upon expression in the *npq2npq4* background lacking Vio and constitutively accumulating Zea (Fig. 7c, d). All these features closely reproduce the properties of LHCSR1 activity in the moss, implying the observed NPQ could be *bona fide* attributed to LHCSR1. LHCSR1 was correctly addressed to the thylakoid membranes with an apparent molecular weight identical to the LHCSR1 of *P. patens*, as observed in SDS-PAGE gels (Fig. 1), implying a correct targeting and processing of the pre-protein encoded by the construct. We then proceeded to identify the factors which determine the level of NPQ activity, including the accumulation in the thylakoids, the availability

of the Zea co-factor and the co-localization with PSII whose fluorescence is quenched during NPQ. The level of LHCSR1 in *A. thaliana npq4* was slightly lower (82.8±1.8%) with respect to the *P. patens psbs-lhcsr2 ko*. Yet, while LHCSR1 is the major contributor to NPQ activity in moss (Alboresi et al. 2010) providing an NPQ activity of 3.2, the NPQ activity in *A. thaliana* was lower. Besides the lower levels of Zea found in *A. thaliana*, this can be explained by (i) a lower level of LHCSR1 activation by a difference in lumen acidification; (ii) a different localization in thylakoids with respect to the PSII antenna system, which is the major fluorescence emitter *in vivo*; or (iii) the lack of one or more interaction partner(s) acting as a docking site for connecting the quenching site within the LHCSR1 to the PSII antenna system. To test the first hypothesis, we proceeded to determine the ΔpH in *npq4*+LHCSR1 and *P. patens psbs-lhcsr2 ko* chloroplasts by the 9-AA quenching method and showed that ΔpH formation is higher in *A. thaliana* with respect to *P. patens*. This would suggest that the lower NPQ in *A. thaliana* might be due to over-acidification of the lumen. LHCSR1, however, was not active at 100µmol photons·m^{-2}·s^{-1} in *A. thaliana*, a light intensity where the ΔpH is comparable to that found in *P. patens.* The highest quenching activity of LHCSR1 in *A. thaliana* was found at 400 and 600µmol photons·m^{-2}·s^{-1}, light intensities where the ΔpH is already much higher in *A. thaliana* in comparison to *P. patens.* Making it unlikely that over-acidification is the reason for the lower quenching activity. Hypothesis (ii) appears to be relevant in determining a reduced NPQ since the membrane fractionation experiment located LHCSR1 in the stroma membranes (Fig. 1c, d), consistent with previous findings in the moss. It should be noted that a large fraction of LHCII is located in moss stroma membranes (Pinnola et al. 2015b), while higher plants show extreme lateral heterogeneity with LHCII being located almost exclusively in the grana (Bassi et al. 1988; Pribil et al. 2014). Thus, interaction between LHCSR1 and PSII antennas appears to be restricted to grana margins, implying that only a low fraction of PSII supercomplexes might be involved. This is likely to decrease the quenching efficiency of *npq4*+LHCSR1 plants with respect to WT *A. thaliana*, with PSBS localized in grana partitions together with PSII antenna (Pinnola et al. 2015b). Hypothesis (iii) is synergic with (ii). In fact, work in *A. thaliana* (Pietrzykowska et al. 2014) and *C. reinhardtii*

(Elrad et al. 2002; Ferrante et al. 2012) has shown that quenching requires specific members of the LHC protein family with which PSBS and LHCSR interact (Girolomoni et al. 2016). It is well possible that one or more LHCSR1-interacting proteins in moss are not conserved in *A. thaliana* and/or that the interaction is partially impaired.

In addition to differences in amplitude, the kinetics of LHCSR1-dependent quenching is also different in transgenic *A. thaliana vs*. *P. patens* in some aspects: first, quenching is activated only upon pre-treatment with high light while in the moss it is evident at the first light exposure of dark-adapted mosses (Fig. S11). Explanation of this behavior is provided by the results of transformed *npq1npq4* and *npq2npq4* genotypes: plants from the former genotype showed no quenching activity due to the absence of Zea, despite LHCSR1 accumulation in the thylakoids. Plants from the latter genotype, the *npq2npq4* which are fully endowed with Zea, not only show a higher quenching activity, but also, a faster activation, i.e. at the first cycle of illumination (Fig. 7c) rather than at the third as in the *npq4* plants expressing LHCSR1. Even though the *npq2npq4* mutant contains 2.5 folds more Zea in comparison to high light adapted *P. patens* (Dall'Osto et al. 2005; Pinnola et al. 2013), the level of NPQ was lower than found in both *P. patens* LHCSR1-only or *A. thaliana* WT, implying that Zea was not the only limiting factor for the quenching activity of LHCSR1 in *A. thaliana*. Interestingly, the kinetics of the de-epoxidation index (DEP) are very similar to the kinetics of quenching in both *A. thaliana* and *P. patens*: in *A. thaliana* the maximal DEP was reached after 10 minutes (Dall'Osto et al. 2017), in *P. patens* a DEP comparable to the maximum of *A. thaliana* was already reached after 1.5 min (Fig. S12). This suggests that there is simply not enough Zea in *A. thaliana* to completely activate LHCSR1, which is consistent with the enhanced activity of LHCSR1 in the *npq2npq4* background where Zea availability was constitutive rather than induced by light exposure. The *lut2npq4* transformed lines, lacking Lut, showed activity in NPQ, meaning that Lut is not an absolute requirement for the *in vivo* quenching in LHCSR1. This is consistent with the recent finding that the major quenching mechanism in isolated LHCSR1 is energy transfer from Chl to S1 state of Zea followed by rapid relaxation to ground state, while transient formation of Lut

radical cation was low (Pinnola et al. 2016). In the *lut2npq4* transformed lines the peak NPQ activity was observed at later times upon each onset of the actinic light. While the NPQ peak was observed after 2 min of light exposure in *npq4*+LHCSR1, the NPQ of *lut2npq4*+LHCSR1 steadily raised till the end of the light phase. This behavior can be explained based on the enhanced Vio content in the L2 binding site, due to compensation for the missing Lut (Pogson et al. 1996; Dall'Osto et al. 2006). Vio is an inhibitor of quenching reactions (Niyogi et al. 1998; Ruban et al. 1998) and replaces Lut in sites L1 and L2 of LHCII proteins (Croce et al. 1999; Dall'Osto et al. 2006). The kinetic difference can be explained with two independent events of xanthophyll exchange: one at site L1, which substitutes Vio for Zea, while the second event is the exchange at site L2 which, are kinetically different in LHC proteins (Morosinotto et al. 2002). Since occupation of L1 site by Lut or Zea is essential for the NPQ activity (Dall'Osto et al. 2006), the onset of NPQ was slower in *lut2npq4*+LHCSR1 with respect to *npq2npq4*+LHCSR1 or *npq4*+LHCSR1 which have Lut in site L1 already in the dark and only need to perform the Vio to Zea exchange in site L2. Future research will need to devise new methods for assessing the xanthophyll composition of LHCSR1 in real time as well as other LHC proteins essential for quenching reactions in plants, mosses and algae since the exchange might be fast and reversible in minutes. It remains to be explained why the *npq2npq4*+LHCSR1 or *npq4*+LHCSR1 show an NPQ kinetic rapidly climbing to a peak and then relaxing or remaining constant during the remaining light period. We suggest this depends on the $\Delta pH + \Delta\psi$ gradient through the thylakoid membrane that appears to be different in *A. thaliana vs P. patens*. 9AA quenching showed a lower ΔpH contribution in *P. patens* and yet LHCSR1 might respond to $\Delta\psi$ as well. Upon transition from dark to excess light, a transient lumen acidification is reached due to the contribution of cyclic electron flow, recycling excess reducing power into over-reduction of plastoquinol and additional proton transport (Munekage et al. 2004). The ΔpH and/or $\Delta\psi$ past transient cyclic burst might be insufficient to sustain full LHCSR1 activation in *A. thaliana*.

Conclusion

We show that heterologous expression of LHCSR1 in *A. thaliana npq4* mutant yields a pigment-binding protein with properties reproducing those of LHCSR1 from the homologous system *P. patens*. The protein is active in NPQ, yet the induction requires sustained light treatment due to the need for Zea build-up. Reasons for a decreased NPQ include (a) insufficient Zea accumulation in *A. thaliana* with respect to *P. patens* for full NPQ activity and (b) the localization of the protein in the stromal membranes of thylakoids which is rich in highly fluorescent LHCII in mosses but not in plants (Pinnola et al. 2015a). The level of quenching in *npq2npq4*+LHCSR1 (endowed with full Lut and Zea levels) recovered up to 70% with respect to *A. thaliana* WT, proving that LHCSR1 can be highly functional in vascular plants. Furthermore, we prove that this system is sensitive to physiological differences which makes *A. thaliana* an excellent organism for the analysis of LHCSR activity. Indeed, we could assess that Lut was not an absolute requirement for *in vivo* quenching in LHCSR1, since quenching activity was obtained in *lut2npq4*+LHCSR1 plants.

The primary target of plants is survival; thus, they favor thermal dissipation over fast growth. But an NPQ mechanism with low activity in moderate light intensity and full activation in extreme stress conditions only, might allow for optimizing both growth and stress resistance. This could be the case for the LHCSR1 protein expressed in the heterologous systems which exhibits an activity, even if low in the WT and higher in the *npq2* background, in stressing conditions. Future work will evaluate the growth performance (productivity) of these plants in different constant light condition as well as in fluctuating light which is the most stressing condition and mimics the natural environment.

Finally, since the NPQ activity of LHCSR1 and PSBS is cumulative, suggesting they have different interaction partners, future work with deletion mutants of specific LHC proteins will pinpoint the interaction partners of LHCSR1 and help us elucidate how PSBS and LHCSR1 evolved.

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Supplementary data

		Fv/Fm			
		Cycle 1	Cycle 2	Cycle 3	Cycle 4
Fig. 3	At WT	0.873±0.007	0.830±0.005	0.807±0.005	0.798±0.008
	At *npq4*	0.874±0.002	0.825±0.002	0.800±0.002	0.787±0.002
	At *npq4*+SR1	0.870±0.003	0.821±0.004	0.793±0.005	0.784±0.005
Fig. 5	Pp *psbs-lhcsr2 ko*	0.839±0.006	0.726±0.010	0.743±0.004	0.722±0.011
	At WT	0.873±0.007	0.830±0.005	0.807±0.005	0.798±0.008
	At *npq4*	0.874±0.002	0.825±0.002	0.800±0.002	0.787±0.002
	At npq4+SR1	0.870±0.003	0.821±0.004	0.793±0.005	0.784±0.005
Fig. 7	*npq1npq4*	0.855±0.004	0.818±0.005	0.799±0.006	0.789±0.010
	npq1npq4 + SR1 L A1	0.854±0.003	0.816±0.007	0.800±0.012	0.787±0.009
	npq1npq4 + SR1 L B1	0.854±0.007	0.814±0.004	0.793±0.015	0.776±0.015
	npq1npq4 + SR1 L C3	0.852±0.005	0.814±0.007	0.794±0.008	0.777±0.008
	npq2npq4	0.858±0.004	0.792±0.007	0.766±0.006	0.756±0.005
	npq2npq4 + SR1 L C1	0.856±0.003	0.797±0.004	0.778±0.004	0.768±0.005
	npq2npq4 + SR1 L C2	0.840±0.005	0.778±0.004	0.761±0.006	0.750±0.006
	npq2npq4 + SR1 L C3	0.844±0.006	0.787±0.010	0.770±0.013	0.761±0.015
	lut2npq4	0.863±0.003	0.824±0.005	0.791±0.004	0.773±0.006
	lut2npq4 + SR1 L A1	0.855±0.003	0.822±0.001	0.789±0.006	0.775±0.004
	lut2npq4 + SR1 L B1	0.854±0.003	0.816±0.003	0.782±0.002	0.771±0.004
	lut2npq4 + SR1 L C1	0.852±0.002	0.818±0.004	0.788±0.003	0.775±0.006

Table S1.
The Fv/Fm values from the different cycles applied to leaves of the measured mutants and different LHCSR1-expressing lines. Standard deviation is reported behind the values.

		Fv/Fm				Fv/Fm
100uE	*npq4*	0.775 ± 0.012		200uE	*npq4*	0.771 ± 0.009
	WT	0.790 ± 0.007			WT	0.791 ± 0.007
	npq4+SR1 L. C1	0.774 ± 0.005			*npq4*+SR1 L. C1	0.772 ± 0.004
	npq4+SR1 L. A5	0.780 ± 0.017			*npq4*+SR1 L. A5	0.771 ± 0.016
	npq4+SR1 L. A1	0.777 ± 0.004			*npq4*+SR1 L. A1	0.769 ± 0.012
400uE	*npq4*	0.778 ± 0.004		600uE	*npq4*	0.772 ± 0.006
	WT	0.792 ± 0.007			WT	0.789 ± 0.006
	npq4+SR1 L. C1	0.773 ± 0.002			*npq4*+SR1 L. C1	0.772 ± 0.007
	npq4+SR1 L. A5	0.777 ± 0.007			*npq4*+SR1 L. A5	0.769 ± 0.009
	npq4+SR1 L. A1	0.772 ± 0.010			*npq4*+SR1 L. A1	0.771 ± 0.009
800uE	*npq4*	0.772 ± 0.007		1000uE	*npq4*	0.768 ± 0.011
	WT	0.794 ± 0.006			WT	0.786 ± 0.004
	npq4+SR1 L. C1	0.771 ± 0.006			*npq4*+SR1 L. C1	0.771 ± 0.008
	npq4+SR1 L. A5	0.772 ± 0.004			*npq4*+SR1 L. A5	0.762 ± 0.004
	npq4+SR1 L. A1	0.775 ± 0.010			*npq4*+SR1 L. A1	0.765 ± 0.015

Table S2.
Fv/Fm of *npq4*+LHCSR1 lines in various light intensities. Three different *A. thaliana npq4*+LHCSR1 lines with high and intermediate NPQ activation (line C1, A1 and A5) were tested in a variety of actinic light intensities. Leaves (n=3) were dark adapted for 45min, pre-treated with 800µmol photons·m^{-2}·s^{-1} of actinic light for 15min and left to relax in the dark for 10min before the NPQ measurement. Each measurement corresponds to one single NPQ cycle of 5min different with different actinic light intensities and 5min dark recovery. The actinic light intensities used were: 100, 200, 400, 600, 800 and 1000µmol photons·m^{-2}·s^{-1} (µE) from a-f respectively. Leaves from *A. thaliana* WT and *npq4* were used as control.

	Chl *a/b*	Car/Chl	V+A+Z	DEP	Neo	Vio	Ant	Lut	Zea	β-car
T_0_Dark										
npq4	2.59 ± 0.08	26.4 ± 1.1	2.6 ± 0.2		4.0 ± 0.2	2.6 ± 0.2		12.5 ± 0.5		6.4 ± 0.7
Line C1	2.65 ± 0.09	27.1 ± 1.4	3.3 ± 0.8		3.9 ± 0.1	3.3 ± 0.8		12.8 ± 0.5		6.2 ± 0.1
Line C3	2.60 ± 0.05	28.5 ± 1.7	3.6 ± 0.8		4.2 ± 0.4	3.6 ± 0.8		13.7 ± 0.9		5.9 ± 0.6
T_{10}_Light										
npq4	2.50 ± 0.07	26.6 ± 0.7	3.4 ± 0.1	0.53 ± 0.07	4.1 ± 0.2	1.4 ± 0.2	0.3 ± 0.1	12.8 ± 0.5	1.7 ± 0.3	6.3 ± 0.2
Line C1	2.59 ± 0.13	26.7 ± 1.2	4.3 ± 0.1	0.44 ± 0.04	3.9 ± 0.3	2.2 ± 0.1	0.4 ± 0.0	12.9 ± 1.0	1.7 ± 0.2	5.7 ± 0.7
Line C3	2.59 ± 0.03	28.1 ± 0.9	4.7 ± 0.2	0.48 ± 0.02	4.1 ± 0.1	2.2 ± 0.2	0.4 ± 0.0	13.4 ± 0.1	2.0 ± 0.1	5.8 ± 0.5
T_{20}_Dark										
npq4	2.60 ± 0.09	26.7 ± 2.7	3.4 ± 0.2	0.53 ± 0.01	4.0 ± 0.7	1.3 ± 0.1	0.6 ± 0.0	12.6 ± 1.6	1.5 ± 0.1	6.7 ± 0.6
Line C1	2.65 ± 0.08	26.4 ± 1.1	4.2 ± 0.4	0.42 ± 0.03	3.6 ± 0.1	2.1 ± 0.3	0.6 ± 0.1	12.6 ± 0.3	1.5 ± 0.1	6.0 ± 0.5
Line C3	2.58 ± 0.06	28.1 ± 2.4	4.7 ± 1.2	0.48 ± 0.02	4.1 ± 0.4	2.2 ± 0.7	0.6 ± 0.1	13.6 ± 1.2	2.0 ± 0.4	5.7 ± 0.3
T_{30}_Light										
npq4	2.48 ± 0.03	27.6 ± 1.4	3.6 ± 0.2	0.63 ± 0.01	4.3 ± 0.3	1.2 ± 0.1	0.3 ± 0.1	13.4 ± 0.6	2.1 ± 0.1	6.3 ± 0.6
Line C1	2.63 ± 0.01	27.1 ± 2.0	4.4 ± 0.6	0.54 ± 0.03	3.8 ± 0.3	1.9 ± 0.4	0.4 ± 0.1	13.0 ± 1.0	2.2 ± 0.2	5.9 ± 0.1
Line C3	2.60 ± 0.07	28.1 ± 1.2	4.4 ± 0.5	0.61 ± 0.01	4.1 ± 0.3	1.5 ± 0.2	0.4 ± 0.0	13.5 ± 0.9	2.5 ± 0.3	6.2 ± 0.2
T_{40}_Dark										
npq4	2.53 ± 0.03	27.6 ± 0.9	3.6 ± 0.2	0.59 ± 0.02	4.2 ± 0.2	1.2 ± 0.1	0.6 ± 0.1	13.2 ± 0.4	1.8 ± 0.2	6.6 ± 0.2
Line C1	2.55 ± 0.06	26.5 ± 1.1	4.0 ± 0.6	0.51 ± 0.02	3.8 ± 0.1	1.6 ± 0.3	0.7 ± 0.1	13.1 ± 0.8	1.7 ± 0.2	5.7 ± 0.6
Line C3	2.54 ± 0.09	27.6 ± 0.8	4.1 ± 0.5	0.56 ± 0.03	4.1 ± 0.1	1.5 ± 0.3	0.7 ± 0.0	13.4 ± 0.8	2.0 ± 0.3	6.1 ± 0.4

Table S3.
HPLC-pigment analysis of *A. thaliana npq4* and two lines expressing LHCSR1 (line C1 and C3). Dark adapted samples were analyzed as well as samples harvested at the end of alternated light/dark cycles of 10 minutes (light intensity: 1200µmol photons·m^{-2}·s^{-1}). Pigments are normalized to 100 Chl molecules and analysis was performed in triplicates. The DEP was calculated by the following formula: (Zea+0.5*Ant)/(Zea+Vio+Ant).

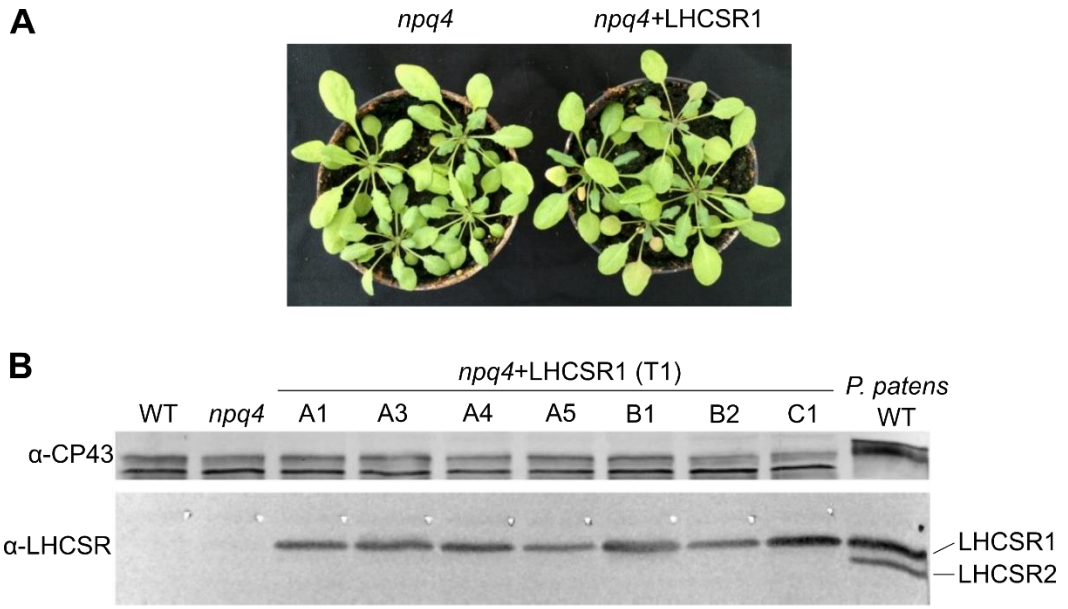

Figure S1.
Biochemical characterization of the *A. thaliana npq4* lines transformed with LHCSR1. The presence of the LHCSR1 subunit was assessed by Western blot. (A) Different lines are shown after 5 weeks of growth. (B) Western blot analysis was performed on total proteins extracted by grinding one leaf disk directly in 100 µL of loading buffer, one tenth of the volume was loaded on an SDS-PAGE. Proteins of WT and *npq4* plants were loaded as a control as well as the equivalent of 1 µg of Chl of thylakoids from WT *P. patens* plants. The primary antibody used for the analysis is indicated on the left side of the membrane while the band corresponding either to *P. patens* LHCSR1 or LHCSR2 is indicated on the right side.

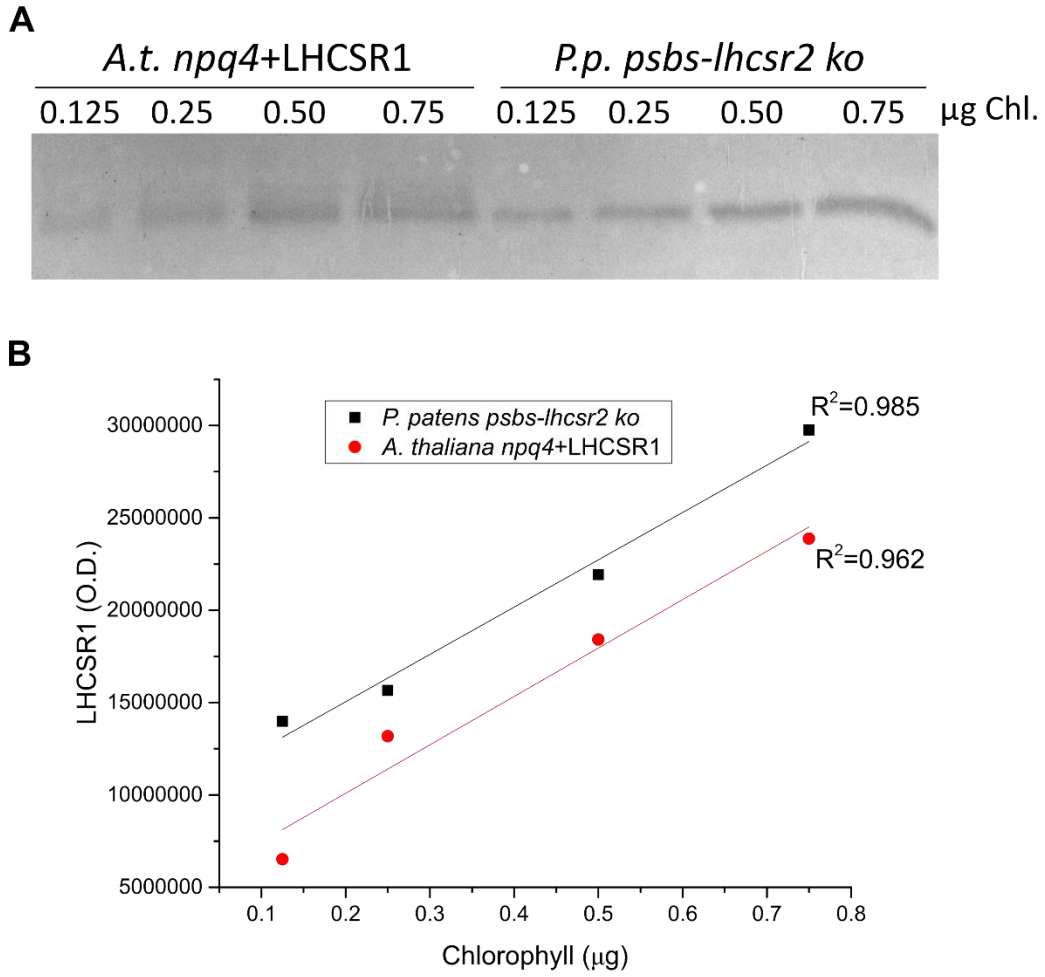

Figure S2.
Comparison of LHCSR1 content in *P. patens psbs-lhcsr2 ko* and *A. thaliana npq4*+LHCSR1. (A) Different concentrations of Chl from whole leaf extracts of *A. thaliana npq4*+LHCSR1 or tissue of *P. patens psbs-lhcsr2ko* were loaded on an SDS-PAGE and blotted against the α-LHCSR antibody. (B) The O.D. of LHCSR1 determined from the western blot was plotted against the Chl concentrations to determine the amount of LHCSR1 in *P. patens* and *A. thaliana*.

Matched peptides shown in ***bold red***.

Chloroplast transit peptide

```
  1 MAIAMSSVSC ISGAKLFSTP AAYQVTRRAG VQRISAVADK VSPDPEVVPP
 51 NVLEYAKGMP GVCAPFPNIF DPADLLARAA SSPRPIKELN RWRESEITHG
101 RVAMLASLGF IVQEQLQDYS LFYNFDGQIS GPAIYHFQQV EARGAVFWEP
151 LLFAIALCEA YRVGLGWATP RSEDFNTLRD DYEPGNLGFD PLGLLPSDPA
201 ERKDMQTKEL NNGRLAMIAI AAFVAQELVS GEEIFVHLFK RLGL
```

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	M	Peptide
41 - 57	1853.0890	1852.0817	1851.9618	64.7	0	K.VSPDPEVVPPNVLEYAK.G
58 - 78	2258.2750	2257.2677	2257.1024	73.3	0	K.GMPGVCAPFPNIFDPADLLAR.A
58 - 78	2274.3090	2273.3017	2273.0973	89.9	0	K.GMPGVCAPFPNIFDPADLLAR.A + Oxidation (M)
79 - 91	1438.9290	1437.9217	1437.8052	81.1	1	R.AASSPRPIKELNR.W
92 - 101	1270.7360	1269.7287	1269.6214	84.5	1	R.WRESEITHGR.V
144 - 162	2226.2130	2225.2057	2225.1343	32.1	0	R.GAVFWEPLLFAIALCEAYR.V
163 - 171	956.6200	955.6127	955.5240	92.9	0	R.VGLGWATPR.S
172 - 202	3449.8720	3448.8647	3448.6059	75.1	1	R.SEDFNTLRDDYEPGNLGFDPLGLLPSDPAER.K
172 - 203	3578.0890	3577.0817	3576.7009	106	2	R.SEDFNTLRDDYEPGNLGFDPLGLLPSDPAERK.D
180 - 202	2487.3920	2486.3847	2486.1601	90.3	0	R.DDYEPGNLGFDPLGLLPSDPAER.K

Figure S3.
The band from LHCSR1 expressed in *A. thaliana* thylakoids was excised. The protein sample was digested with trypsin overnight. The peptides obtained were analyzed using a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (KRATOS Analytical, Shimadzu corporation, Japan). Database search was performed by the Mascot wizard from www.matrixscience.com. The protein identification results were obtained from the primary mass spectrum of the peptides produced after enzymatic hydrolysis. Search parameters: Trypsin enzymatic solution, set two missed cut sites. The alkylation of cysteine is set to a fixed modification, and the oxidation of methionine as a variable modification. The database used for the authentication was NCBInr.

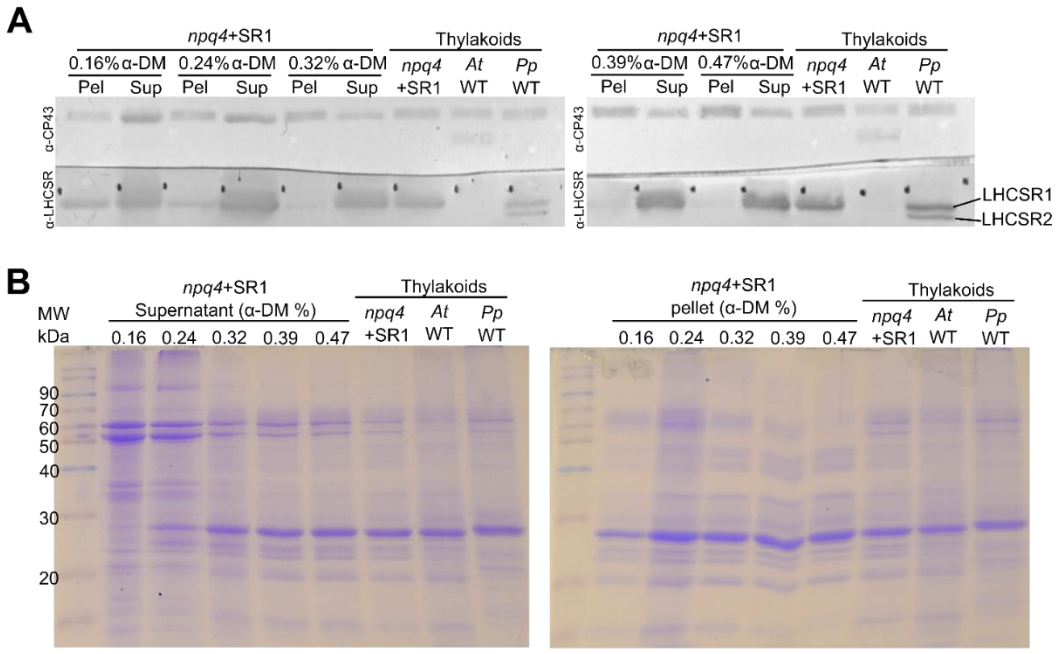

Figure S4.
Grana and stroma separation from *npq4*+LHCSR1 thylakoids by fractionation with different α-DM concentrations. (A) Western blot analysis of LHCSR1 distribution of the pellet and supernatant upon fractionation of isolated thylakoids with different concentrations of α-DM (0.16-0.47%). The pellet (Pel) is enriched in the grana fractions, while the supernatant (Sup) mostly contains stroma fractions. Fractions and thylakoids were loaded on SDS-PAGE gels. Equal amounts of thylakoids from *A. thaliana npq4*+LHCSR1 (*npq4*+SR1), *A. thaliana* WT (*At* WT) and *P. patens* WT (*Pp* WT) were loaded as controls. All samples were loaded on a Chl basis of 0.5µg. (B) Coomassie staining of thylakoids and fractionated thylakoid membranes, samples were loaded on a Chl basis of 2µg.

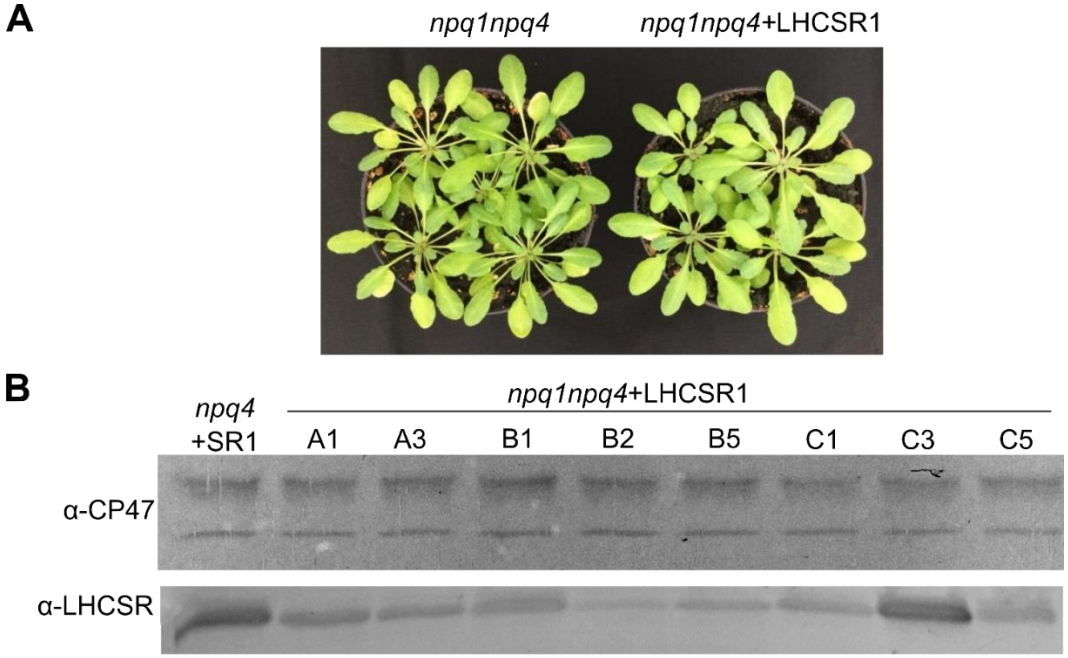

Figure S5.
Immunological screening of *npq1npq4*+LHCSR1 transformed lines. (A) *A. thaliana* transgenic lines were selected on agar plates and then transferred in pots in a short-day photoperiod growth chamber (right). Control *npq1npq4* plants of the same age were also grown in the same conditions (left). (B)Western blot analysis of 8 independent transformed lines from the T2 generation and *npq4*+LHCSR1 as a control.

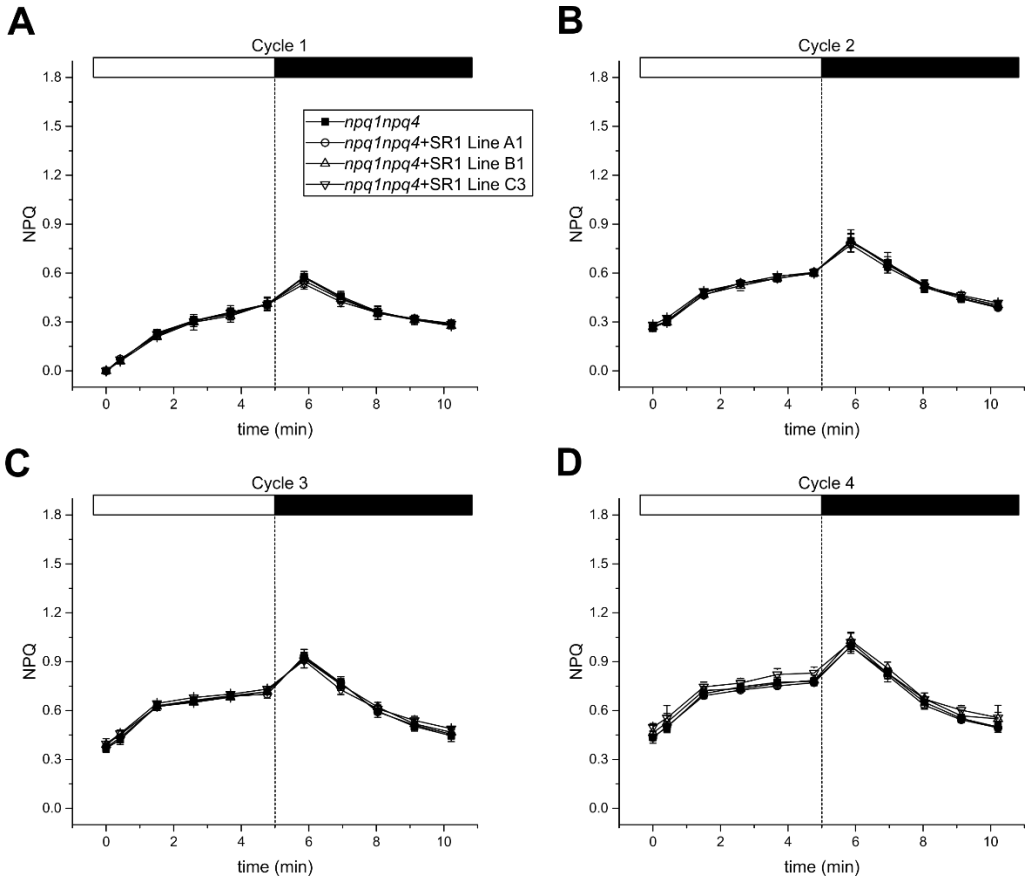

Figure S6.

NPQ measurements in *A. thaliana npq1npq4* transformed with LHCSR1 using fluorescence video-imaging (n=3). NPQ of Chl fluorescence was measured in leaves taken from 4-5-week old plants. Four successive NPQ cycles were measured, protocol: 5 minutes of actinic light treatment (1200µmol photons·m^{-2}·s^{-1}) followed by 5 min of dark recovery. The four cycles are presented by A-D respectively.

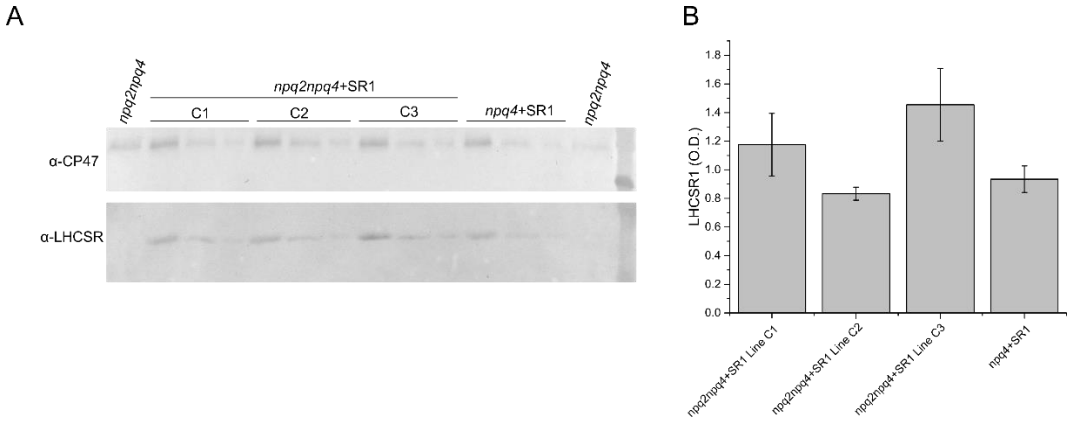

Figure S7.
Immunological screening and immuno-titration of *npq2npq4*+LHCSR1 transformed lines. (A) Western blot analysis of was performed on total protein extracts. Chl concentration was determined, and different amounts were loaded on the gel. Proteins of non-transformed (*npq2npq4*) and transgenic *npq4*+LHCSR1 plants were loaded as controls. (B) The O.D. of LHCSR1 was determined from the western blot was plotted against the Chl concentrations to determine the amount of LHCSR1 in the *npq2npq4* transformed lines in comparison to *npq4*+LHCSR1.

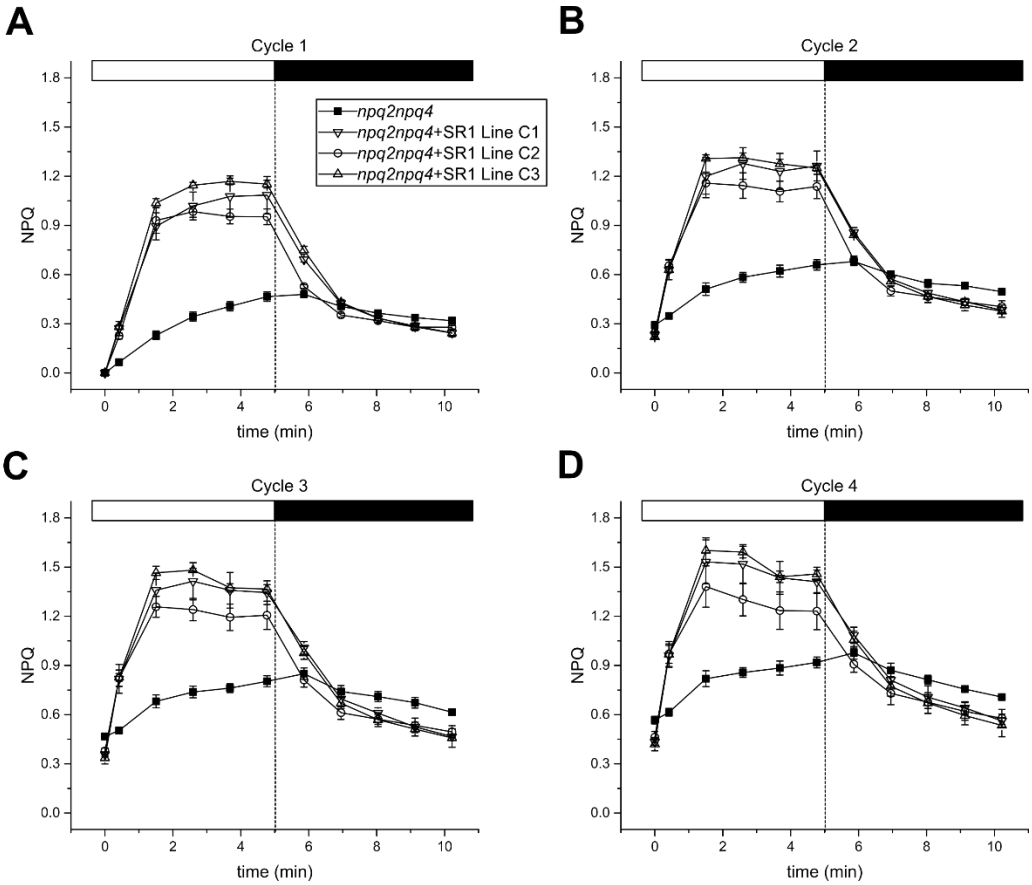

Figure S8.

NPQ measurements in different *A. thaliana npq2npq4* transformed with LHCSR1 using fluorescence video-imaging (n=3). NPQ of Chl fluorescence was measured in leaves taken from 4-5-week old *npq2npq4* and 3 transformed lines. Four successive NPQ cycles were measured; 5 minutes of actinic light treatment (1200µmol photons·m^{-2}·s^{-1}) followed by 5 min of dark recovery. The four cycles are presented by A-D respectively.

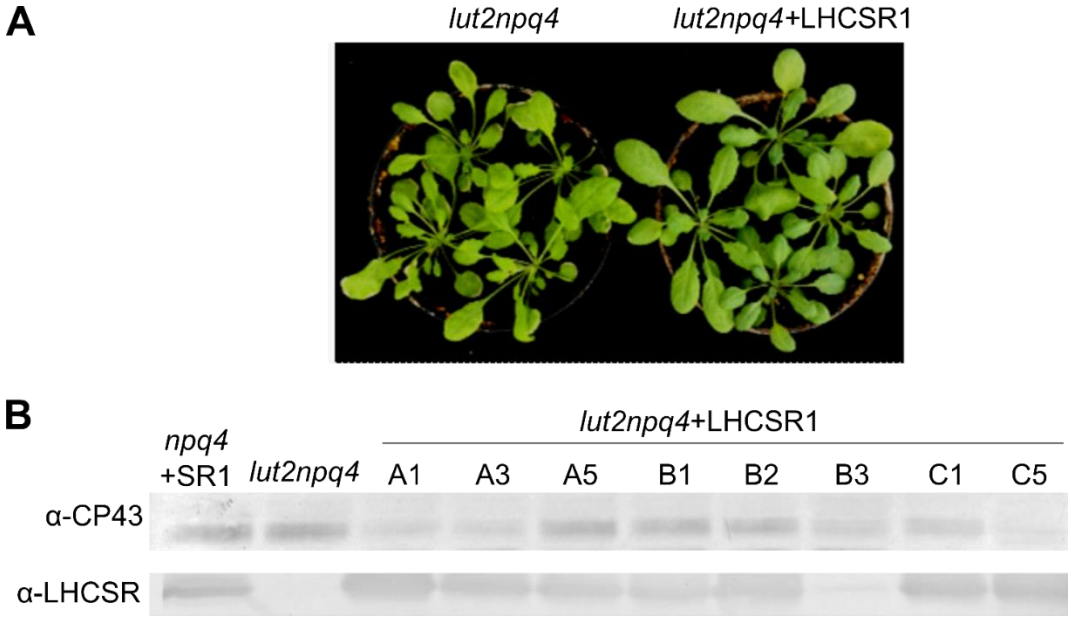

Figure S9.
Immunological screening of *lut2npq4*+LHCSR1 transformed lines (n=3). (A) *A. thaliana* transgenic lines were selected on agar plates supplemented with hygromycin-B. Control *lut2npq4* plants of the same age were also grown in the same conditions. (B)Western blot analysis of 8 independent plant lines together with *lut2npq4* and *npq4*+LHCSR1 as controls.

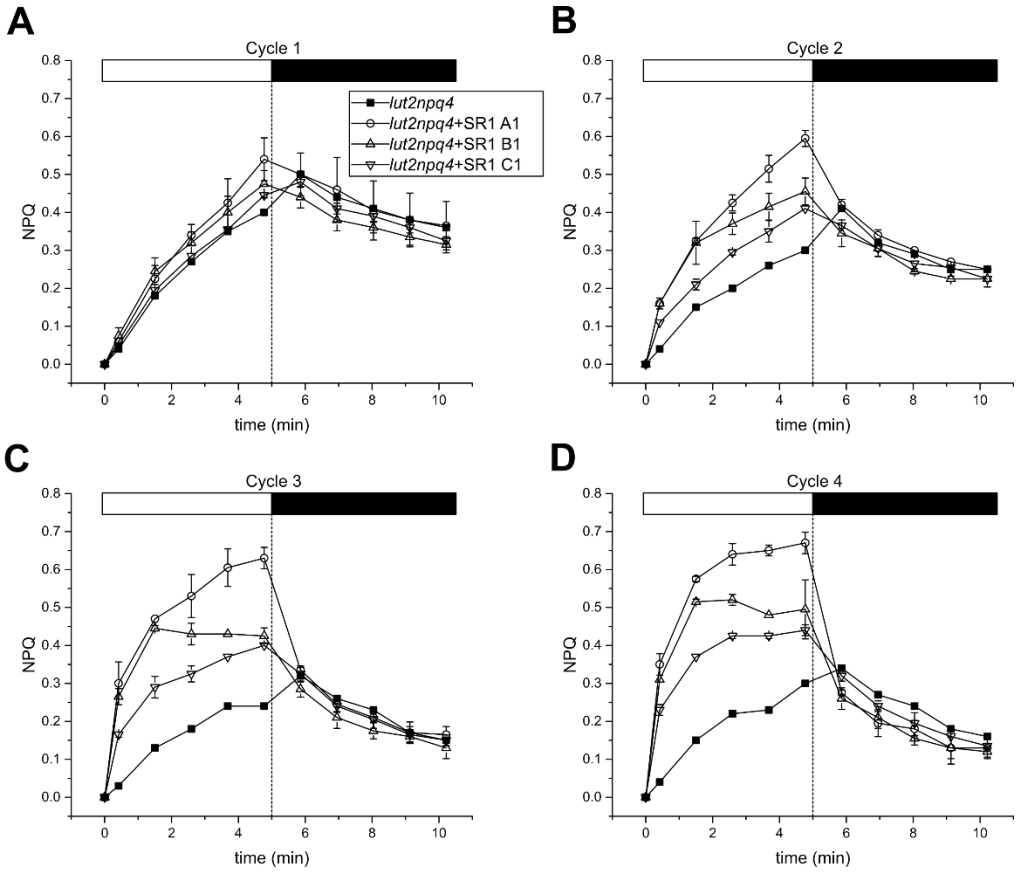

Figure S10.
NPQ measurements in different *A. thaliana lut2npq4* transformed with LHCSR1 using fluorescence video-imaging (n=3). NPQ of Chl fluorescence was measured in leaves taken from 4-5-week old plants. Four successive NPQ cycles were measured, protocol: 5 minutes of actinic light treatment (1200µmol photons·m^{-2}·s^{-1}) followed by 5 minutes of dark recovery. The four cycles are presented by A-D respectively.

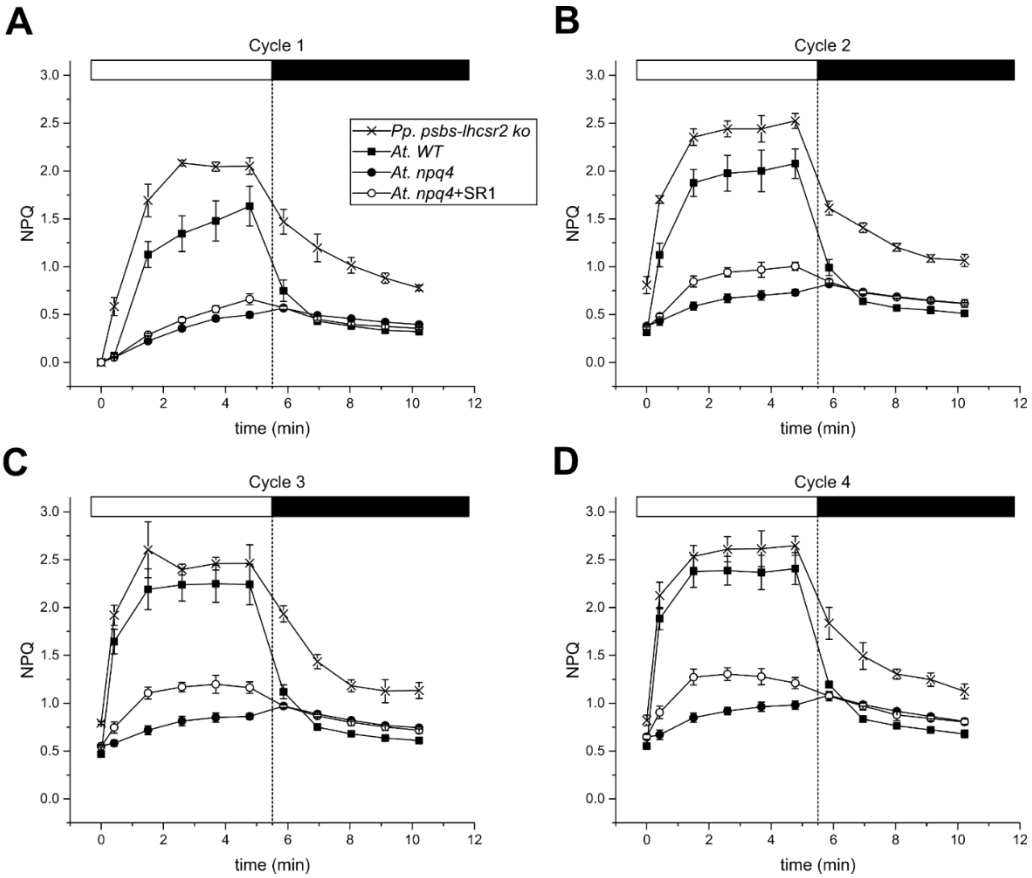

Figure S11.
NPQ measurements (n=4) in *A. thaliana WT, npq4, npq4*+LHCSR1 and *P. patens psbs-lhcsr2 ko (n=3)*. Measurements were performed with video-imaging at 1200µmol photons·m^{-2}·s^{-1} with 4 cycles of 5min light and 5 min dark, figure A-D respectively.

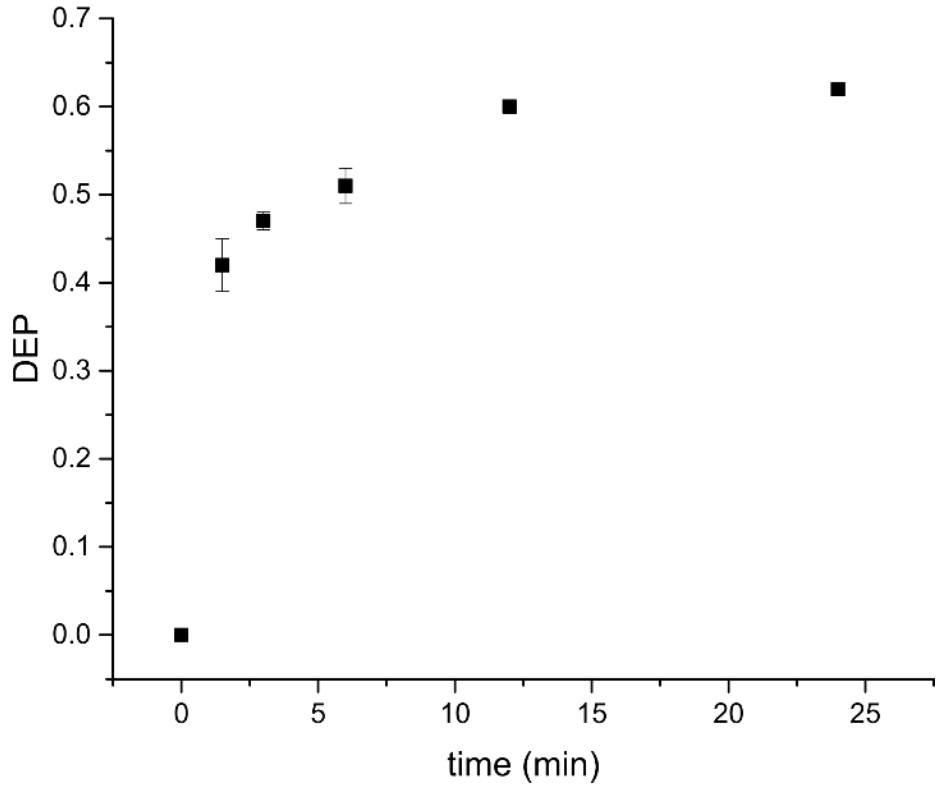

Figure S12.
Time course of the Deepoxidation index (DEP) in *P. patens* (n=3). Plants were subjected to 850µmolphotons·m^{-2}·s^{-1} and samples were taken and frozen in liquid nitrogen after different time intervals to be analyzed by HPLC. The DEP was calculated by the following formula: (Zea+0.5*Ant)/(Zea+Vio+Ant).

Chapter 3

Determining the interaction partner of LHCSR1 from the moss *P. patens* by heterologous expression in *A. thaliana* mutants

Abstract

Photosynthesis is the process where light is converted and stored into chemical energy. However, plants, mosses and algae experience huge fluctuations in light during the day. Excess light can be extremely damaging and needs to be safely dissipated, the energy dissipation is regulated by either PSBS or LHCSR in plants and algae respectively which respond to the chloroplast lumen acidification induced by excess light. This energy dissipation is called energy quenching (qE) and the precise mechanism is not well understood. It is known that qE requires a chlorophyll/xanthophyll subunit to catalyse the dissipation, however PSBS does not bind any pigments and quenching must therefore occur in another subunit, where quenching reactions are catalysed, most likely Lhcb4 (also known as CP29) and LHCII. LHCSR on the other hand binds pigments and is therefore most likely not only the activator, but also the site of quenching. Interestingly the evolutionary intermediate *Physcomitrella patens* expresses both functional PSBS and LHCSR and previously we've been able to partly restore qE in *Arabidopsis thaliana npq4* mutant plants, lacking PSBS and therefore qE, with LHCSR1 from *P. patens.* However, the precise interaction partners of LHCSR are not known and by complementing different mutants of *A. thaliana*, lacking besides PSBS different antenna proteins, with LHCSR1 we were able to ascertain that neither Lhcb4 nor Lhcb6 is involved in the quenching activity of LHCSR1. However, due to the low expression levels of LHCSR1 in the absence of *lhcb5* we were unable to determine whether the interaction partner is Lhcb5 and/or LHCII as previously suggested.

Introduction

Photosynthetic organisms need light to survive, but excess light can be damaging. During the day they face a huge diversity of light intensities and are therefore required to balance between light-harvesting, in low light conditions, and safely dissipating the excess energy in high light conditions. The differences in light intensity are not only caused by the rising and setting of the sun, but changes induced by canopy movement or clouds passing before the sun play an even more vital role due to the shorter timescales in which they occur. Therefore, photosynthetic organisms have developed a wide array of photoprotection mechanisms to safely dissipate excess energy when in high light conditions. The main photoprotective mechanism is called Non-Photochemical Quenching (NPQ), a process that safely dissipates the excess energy in the form of heat, to avoid the production of singlet oxygen, a harmful reactive oxygen species (ROS).

NPQ comprises several components that are active in different time scales. The two main components are: qE (energy dependent quenching) and qZ (zeaxanthin dependent quenching), which are both activated upon the acidification of the thylakoid lumen that occurs in high light conditions (Horton et al. 1996; Kramer et al. 1999; Kanazawa and Kramer 2002). In excess light, there is an increase in the linear electron transport, thereby increasing the proton transport over the thylakoid membranes. Since the dynamics of the Calvin-Benson cycle are slower with respect to the electron flow, leading to a lack of substrate for the ATPase (ADP and Pi), the excess light induces an acidification of the lumen and an increased ΔpH over the thylakoid membranes, thereby activating the qE and qZ. The first component of NPQ, qE, is activated and deactivated in 1-2 minutes, and the acidification is responsible for the protonation of specific amino acids of LHCSR in algae or PSBS in plants, which are essential for the activation of NPQ (Li et al. 2004; Ballottari et al. 2016). The second component (qZ), depends on the conversion of violaxanthin into zeaxanthin, mediated by the enzyme violaxanthin de-epoxidase, which takes around 8-10 min for the violaxanthin-pool to be fully converted. However, the relaxation of qZ takes in general much longer than that of qE and is therefore often grouped with qI in chlorophyll fluorescence measurements. qI stands for

photoinhibition and comprises the inactivation of the PSII reaction centres, caused by the damaging effects of ROS, and the long-term adaptation to the light-environment effectively decreasing the antenna size of the photosystems (Brooks et al. 2013). Another component, known as qT or state transitions, is responsible for the movement of LHCII from PSII to PSI or the other way around, to balance the energy absorption between the two photosystems (Allorent et al. 2013). The last component is only found in plants and is referred to as qM, which represents the chloroplast movement (Cazzaniga et al. 2013).

Although the precise mechanism of qE is still the object of a lively debate, a common ground for all theories is the involvement of pigment-pigment interactions between chlorophylls and xanthophylls (Young and Frank 1996; Holt et al. 2005; Bode et al. 2009). Since PSBS does not contain pigments (Dominici et al. 2002) it relies upon an interaction with other antenna proteins, namely LHCII and Lhcb4 (CP29), where the actual quenching occurs (Dall'Osto et al. 2017). LHCSR on the other hand is not only the activator (Liguori et al. 2013; Ballottari et al. 2016), but most likely also the site of quenching due to the fact that it binds Chl a, lutein and violaxanthin (Bonente et al. 2011; Pinnola et al. 2015b) and the short lifetime it has in the quenched state (Bonente et al. 2011; Pinnola et al. 2017). LHCSR is present in all algae, except for the red algae and glaucophytes, while PSBS occurred first in the green algae and seems to have replaced LHCSR upon land colonization (Alboresi et al. 2010). The exception to this rule is the moss *Physcomitrella patens*, an evolutionary intermediate between green algae and plants, expressing LHCSR and PSBS which are both functional in NPQ (Alboresi et al. 2010). Since LHCSR and PSBS evolved alongside each other, they most probably have different interaction partners. While the interaction partners of PSBS are known (Dall'Osto et al. 2017), the interaction partners of LHCSR are still being investigated. Single particle electron microscopy showed LHCSR-dimers from *C. reinhardtii* interacting with both Lhcb5 and LHCII (Semchonok et al. 2017). While Fluorescence measurements showed that LHCSR in *P. patens* and *C. reinhardtii* quenches both PSII and PSI, most likely via an interaction with LHCII that is highly abundant in both grana and stroma in both mosses and algae (Pinnola et al. 2015a; Girolomoni et al. 2019). Previously LHCSR1 from *P. patens* has been expressed in

both *N. tabacum* and *A. thaliana* (Pinnola et al. 2015b; Dikaios et al. 2019), where it has been shown to be active in NPQ and can partly complement the level of NPQ in a PSBS-lacking *A. thaliana* mutant (*npq4*). Since LHCSR1 is active in *A. thaliana* and because there is a huge library of *A. thaliana* mutants, including knockouts of almost all the antenna complexes, it is the ideal system to study the interaction partner of LHCSR1. In this work we've transformed several lines lacking different monomeric antenna complexes with LHCSR1 and identified an essential monomeric antenna complex for both NPQ and stability of the LHCSR1 protein.

Materials and Methods

Growth conditions

A. thaliana plants (ecotype *Columbia*) were grown in controlled conditions, stable temperature 23°C during the day and 20°C during the night, in a short-day light regime of 8 hours light and 16 hours dark with a light intensity of 150 µmol photons·m^{-2}·s^{-1}. For flowering, plants were put in long day conditions; 12 hours of light and 12 hours of dark.

Transformation and selection

Transformation was performed according to the floral-dip method (Clough and Bent 1998), using the *A. tumefaciens* GV3101 transformed with pH7WG2.0 harbouring *LHCSR1* from *P. patens* under the control of a 35S-promoter (Dikaios et al. 2019). Selection was performed on Murashige-Skoog medium supplemented with 25µg/mL Hygromycin. To kill the remaining *Agrobacteria*, 100µg/mL Carbenicillin was added to the medium in addition to the Hygromycin for the T1 generations.

SDS-PAGE and immunoblot analysis

Leaf disks were taken and immediately frozen in liquid nitrogen before being homogenized in Laemmli buffer (62.5mM Tris pH6.8, 10%glycerol, 5%SDS, 5% β-mercapto-ethanol). Samples were loaded on a 15% (w/v) acrylamide gel (75:1, acrylamide:bisacrylamide) with 6M of Urea. Proteins were then transferred to a

PVDF membrane and developed with specific antibodies against LHCSR, CP43 and Lhcb5 (CP26), produced in the lab.

Thylakoid isolation and fractionation

Isolation of thylakoid membranes were purified from protonemal tissue of *P. patens* plants following the same protocol used for seed plants (Bassi and Simpson 1987). Thylakoids were fractionated by solubilisation with α-DM as described previously (Morosinotto et al. 2010). Unsolubilized thylakoids were pelleted by centrifugation at 3500g for 5 min. Grana membranes were instead pelleted with a further 30-min centrifugation at 40,000g, whereas solubilized complexes and stroma membranes remained in the supernatant.

Deriphat-PAGE and Mass-Spec Analysis

The different fractions (pellet and supernatant) obtained α-DM solubilization were analysed by non-denaturing Deriphat-PAGE as described by (Peter et al. 1991). Green bands were excised from the gel and digested with trypsin as previously described (Trotta et al. 2016). The eluted peptides were identified by nanoscale liquid chromatography/electrospray ionization tandem mass spectrometry (nLC/ESI-MS/MS) using a Q-Exactive instrument (Thermo Scientific). The MS/MS spectra were analysed against TAIR10 as previously described (Pinnola et al. 2018).

Fluorescence measurements

Chl fluorescence was measured in leaves after a dark adaptation of 30min using a closed FluorCam FC 800MF Video-imaging system (Photon Systems Instruments, Czech Rep.) or the Dual-PAM-100 fluorometer (Walz, Germany). Saturating pulses were 4000μmol photons·m^{-2}·s^{-1} and actinic light of 1200μmol photons·m^{-2}·s^{-1} used. Fv/Fm and NPQ parameters were calculated as (Fm−Fo)/Fm and (Fm−Fm′)/Fm′ respectively.

Reverse Transcription and qPCR by Real Time PCR

mRNA was isolated from *A. thaliana* leaves using the Nucleozol protocol from Macherey-Nagel. Leaves (50mg) were taken and immediately frozen in liquid

nitrogen before grinding and homogenizing in 750uL Nucelozol. After the addition of 300uL RNase-free water, samples were incubated for 10min at room temperature before centrifugation at 12.000g for 15min. Pellet was discarded, and RNA precipitated with the addition of one volume of isopropanol to the supernatant followed by centrifugation at 4˚C. Then mRNA was washed two times with 75% ethanol (diluted with RNase-free water) and finally resuspended in 30uL of RNase-free water. Before reverse transcription, 2µg of mRNA was treated with DNase using Promega RQ1 RNase-free Dnase. Reverse transcription was performed using the Promega GoScript Reverse Transcriptase kit, with Oligo(dT). The qPCR was performed using the GoTaq qPCR Mastermix with the addition of the CXR-dye as a passive reference. Measurements were performed in a 96wells plate using the Applied Biosystems StepOnePlus Real-Time PCR system.

Results

Expression of LHCSR1 from *P. patens* in *A. thaliana npq4NoM*

The *A. thaliana* mutant *npq4NoM,* lacking not only PSBS (*npq4*), but also the monomeric antenna complexes (*NoM*); Lhcb4 (CP29), Lhcb5 (CP26) and Lhcb6 (CP24) (Dall'Osto et al. 2017), was transformed with LHCSR1 from *P. patens* under the 35S promoter using the *Agrobacterium*-mediated floral dip method (Clough and Bent 1998). Twenty-one lines survived the selection on Hygromycin and were then transferred to the soil. Plants were allowed to grow for a few weeks before the protein extracts of the leaves were screened using western blotting with the α-LHCSR (Pinnola et al. 2013) and the CP43 antibodies. As controls, *npq4* transformed plants expressing LHCSR1 (*npq4*+LHCSR1) (Dikaios et al. 2019) and *npq4NoM* background lines were loaded, see Fig. 1A. Even though the transformed lines were resistant to Hygromycin, only a few lines were found to express LHCSR1.

The level of the LHCSR1 protein was far lower in the transformed *npq4NoM* lines in comparison to the control line (*npq4*+LHCSR1). To verify if the transformed lines did contain the transgene and were producing mRNA, we checked the levels of mRNA by Reverse Transcriptase quantitative PCR (RT-qPCR), see Fig. 1B. These lines did express the mRNA, albeit in much lower amounts than the

npq4+LHCSR1. This low expression of both the mRNA and protein in the *npq4NoM* lines has been confirmed in multiple separate transformation events and different rounds of selection, all with the same result.

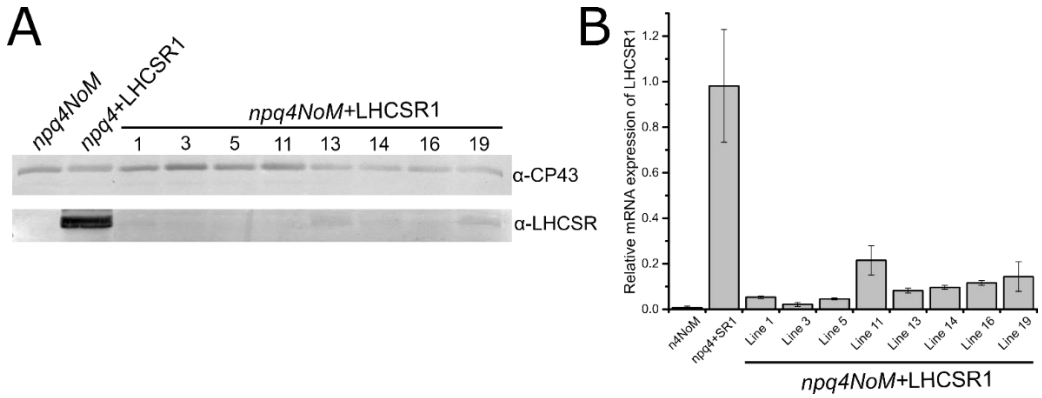

Figure 1 Western blot and mRNA expression levels of the *npq4NoM* lines expressing LHCSR1 in comparison to npq4+LHCSR1

Analysis of LHCSR1 protein level and level of mRNA in the transformed npq4NoM lines. A) Western blots of the highest LHCSR1 expressors in the transformed npq4NoM lines. B) Level of mRNA expression in the transformed lines, normalized to the npq4+LHCSR1 line.

Transformation of LHCSR1 in *A. thaliana npq4Lhcb4* and *npq4Lhcb6*

Since we only observed the absence of LHCSR1 expression in the transformed *npq4NoM* lines, see also Dikaios et al 2019, we hypothesized that LHCSR1 requires one of the monomeric antenna proteins for the stable binding and accumulation.

To identify which of the monomeric antenna complexes is responsible for the stable expression and accumulation of LHCSR1, we proceeded with the transformation of two mutant knockouts in either Lhcb4 or Lhcb6, *npq4Lhcb4* and *npq4Lhcb6* respectively. Important to note is that the absence of Lhcb4 in the *npq4Lhcb4* mutant also leads to the absence of Lhcb6 (de Bianchi et al. 2011).

Both *npq4Lhcb4* and *npq4Lhcb6* transformed lines show protein expression levels comparable to the *npq4*+LHCSR1, see Fig. 2A and C. Indicating that neither Lhcb4 nor Lhcb6 is essential for the stability of LHCSR1. The recovery and the kinetics of NPQ in the *npq4Lhcb6* mutant were similar to those observed in *npq4*+LHCSR1 with a maximum NPQ reached at two minutes after which the NPQ level drops (Dikaios et al. 2019). The NPQ traces of the *npq4Lhcb4* transformed lines are somewhat different from the *npq4* and *npq4Lhcb6* transformed lines, Fig. 2B and

D. There is no maximum at two minutes, and neither is there a decrease in the NPQ after the maximum has been reached. Instead the maximal NPQ is reached around 3 minutes and the level of NPQ stays stable until the actinic light is switched off.

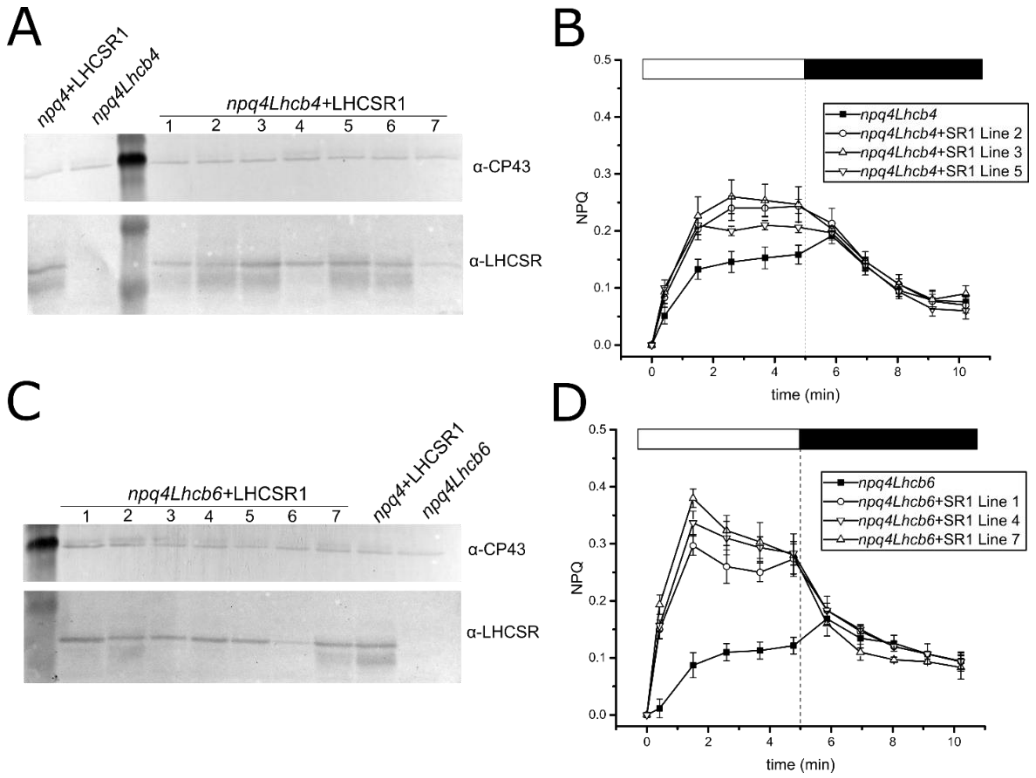

Figure 2. Protein expression of LHCSR1 and NPQ measurements of transformed *npq4Lhcb4* and *npq4Lhcb6, npq4*+LHCSR1 and background strains.

Analysis of the LHCSR1 protein level and NPQ kinetics after 10min of pre-adaptation to high light and 10min of dark relaxation in the transformed lines (npq4Lhcb4 and npq4Lhcb6). A) Western blots of the highest LHCSR1 expressors in the transformed npq4Lhcb4 lines. B) NPQ kinetics of three LHCSR1 transformed npq4Lhcb4 lines. C) Western blots of the highest LHCSR1 expressors in the transformed npq4Lhcb6 lines. D) NPQ kinetics of three LHCSR1 transformed npq4Lhcb6 lines.

Transformation of LHCSR1 in *A. thaliana npq4Lhcb5*

Since both Lhcb4 and Lhcb6 are not involved in the stability of LHCSR1, we transformed a knockout mutant lacking not only PSBS, but also the last monomeric antenna Lhcb5, *npq4Lhcb5*. In this case the same phenotype was observed as in the *npq4NoM* transformed lines, the accumulation of LHCSR1 was very low as was the expression of the mRNA, see Fig. 3. This was also the case in the *npq4NoM* lines, which has been confirmed in multiple separate transformation events and in several different rounds of selection.

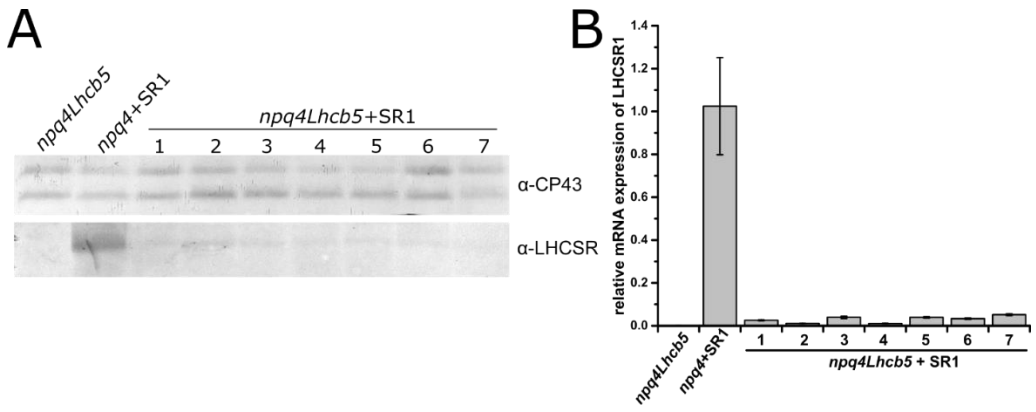

Figure 3 Western blot and mRNA expression levels of the *npq4Lhcb5* lines expressing LHCSR1 in comparison to the *npq4*+LHCSR1.

Analysis of LHCSR1 protein level and level of mRNA in the transformed npq4Lhcb5 lines. A) Western blots of the highest LHCSR1 expressors in the transformed npq4Lhcb5 lines. B) Level of mRNA expression in the transformed lines, normalized to the npq4+LHCSR1 line.

Interaction partners of LHCSR1 in *P. patens*

Since in *P. patens* LHCII is more homogeneously distributed between the grana and the stroma under normal growth conditions it has been proposed that LHCII is one of the interaction partners of LHCSR1 (Pinnola et al. 2015a), we aimed to narrow down the LHCII-isoforms involved in the interaction with LHCSR1 by separating grana and stroma fractions using α-DM in *P. patens*, see Fig 4A, and using Mass-Spec analysis to identify several different LHCII-isoforms that are more abundant in the stroma in comparison to the grana, see Fig. 4B & C.

The Lhca1-3 isoforms are as expected more dominantly found in the supernatant, in which the stroma membranes are enriched and thus contain PSI. The pellet on the other hand is more enriched in the grana partitions and thus in the monomeric antenna Lhcb4 and Lhcb5 bound to PSII, see Fig 4B.

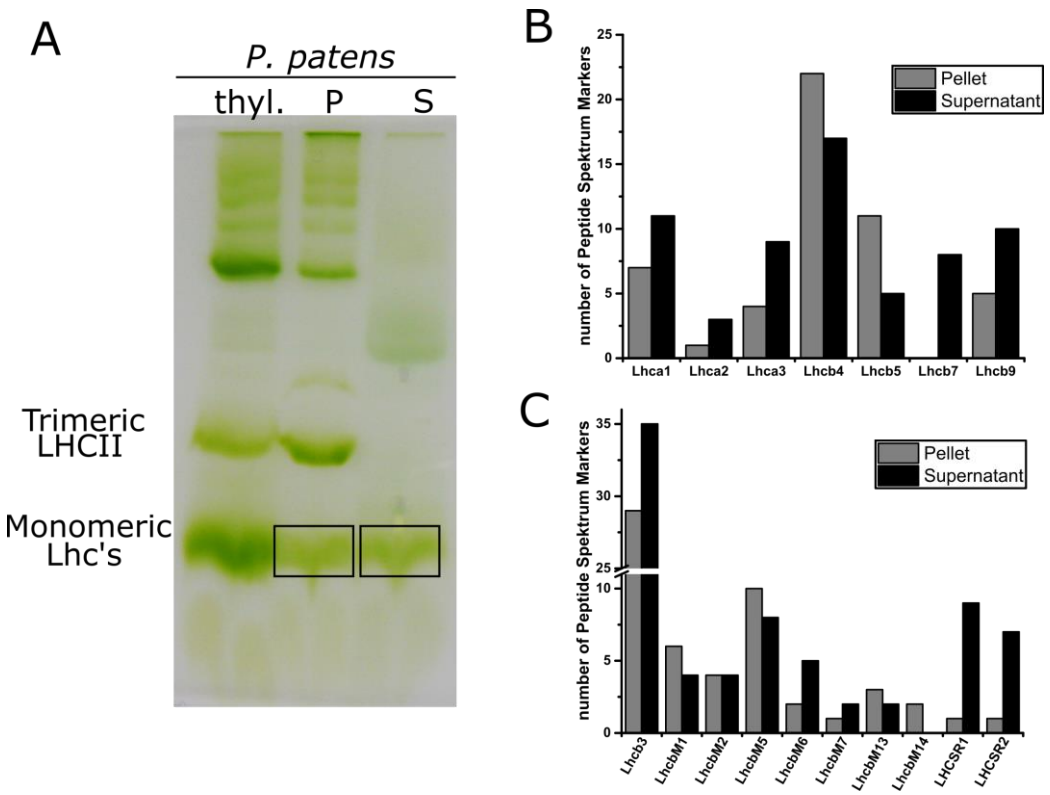

Figure 4 Green gel of thylakoids from *P. patens* for fractionation and Mass-Spec analysis

Green gel (4-16%) on thylakoids isolated from P. patens and fractionations obtained by α-DM solubilisation. Pellet (P) are enriched in grana partitions while the supernatant (S) is enriched in the stroma membranes. A) Green gel with the bands indicated that were used for the Mass-Spec analysis. B and C) indicates the number of Peptide Spektrum Markers for the different proteins found in the different samples (Supernatant or Pellet)

While Lhcb9, a protein highly similar to Lhcb proteins normally attached to PSII while having a red-shifted absorbance spectrum comparable to antennae normally associated with PSI, previously thought to connect to PSII (Alboresi et al. 2011), but later identified to attach to PSI (Pinnola et al. 2018), was found to be more abundant in the supernatant as suspected.

LHCSR1 and LHCSR2 are more abundant in the supernatant together with Lhcb3, LhcbM6 and LhcbM7, while the other isoforms are evenly distributed or more abundantly found in the pellet, see Fig. 4C.

Discussion

The quenching mechanism of LHCSR1 proposes an interesting dilemma; LHCSR1 is located in the stroma membranes while being an active PSII-quencher from

which it is physically separated. In this chapter we tried to answer the question of how LHCSR1 is interacting with PSII by analysing NPQ kinetics and protein expression in transformed *A. thaliana* lines lacking not only PSBS, but also different antenna proteins.

Four different lines lacking specific antenna complexes were transformed with LHCSR1, the first line *npq4NoM* lacks all the monomeric antenna complexes of PSII (Lhcb6, Lhcb5 and Lhcb4) and thus only contains the PSII reaction centre together with the LHCII complexes. The other three lines were lines missing the specific genes encoding the monomeric proteins (*npq4Lhcb6*, *npq4Lhcb5* and *npq4Lhcb4*).

Expression of LHCSR1 in the *npq4NoM* and *npq4Lhcb5* showed the same trend, the level of LHCSR1, in both protein and mRNA, was far lower and hardly detectable in the transformed lines, while in *npq4Lhcb6* and *npq4Lhcb4* transformed plants several lines were found to express LHCSR1 in comparable levels to the highest expressor of the *npq4*+LHCSR1 lines. Furthermore, the *npq4Lhcb6* and *npq4Lhcb4* transformed lines showed recovery of NPQ, with the *npq4Lhcb6*+LHCSR1 lines showing similar NPQ kinetics as found in *npq4*+LHCSR1. The *npq4Lhcb4* lines showed a slightly different recovery of NPQ. A possible explanation is that the absence of Lhcb4 disrupts the structure of the PSII complex, while the macro-structure of PSII is maintained if either Lhcb6 or Lhcb5 are absent (de Bianchi et al. 2008, 2011). These results lead to the conclusion that neither Lhcb4 nor Lhcb6 are important for the quenching activity of LHCSR1 with PSII in *A. thaliana*. The fact that LHCSR1 is not interacting with Lhcb4 is interesting since it has been shown that Lhcb4 is essential for the NPQ activity of PSBS (Dall'Osto et al. 2017). This might be explained by the current knowledge that LHCSR and PSBS evolved alongside each other (Alboresi et al. 2010) and that they therefore have evolved to interact with different partners. Furthermore, since both LHCSR and PSBS were found to be expressed and active in NPQ in the green alga *C. reinhardtii* (Tibiletti et al. 2016) and since green algae lack Lhcb6, it is likely that neither LHCSR nor PSBS interact with Lhcb6. Therefore an interaction of LHCSR1 with either Lhcb5 or LHCII (or both) is very likely and has previously

been observed by electron microscopy in *C. reinhardtii* where LHCSR3 can be found to bind to Lhcb5 and LHCII (Semchonok et al. 2017). Furthermore it has been shown that LHCSR1 in *P. patens* is quenching both PSII and PSI which led to the hypothesis that LHCSR1 in *P. patens* is interacting with PSII via LHCII due to the more even distribution of LHCII between the grana and the stroma (Pinnola et al. 2015a). This hypothesis is consistent with the recent finding that under quenching conditions LHCSR1 in *C. reinhardtii* is involved in transfer of excitation energy from LHCII to PSI instead of PSII (Kosuge et al. 2018).

In this work we found that LHCSR1 is not stably accumulating in *A. thaliana* if the gene encoding *lhcb5* is missing. Whether this is due to a problem with the background of the *lchb5* mutant that is less able to express proteins in general or if this is related with a specific regulation of the *lhcsr1* expression is still unclear. Important to note is that transformation of the *lhcb5*-mutant with the native gene encoding *lhcb5* did not result in any recovery of Lhcb5 (data not shown). On the other hand, it would be strange that the absence of *lhcb5* regulates the transcription level of *lhcsr1* in this particular case since the introduced *lhcsr1*-gene is under the control of the 35S promoter. However, it is possible that *lhcb5* is involved in the LHCSR1 accumulation in the thylakoid membranes via different pathways, such as a correct stabilisation of the mRNA of *lhcsr1* or the correct import into the chloroplasts. Recently it has been shown that if *lhcb5* is absent in *P. patens*, the transcription level of *lhcsr1* is reduced (Peng et al. 2019). Although nothing is known about the protein level of LHCSR1 in this mutant, the level of NPQ in the *lhcb5* knockout is similar to that of the *lhcsr1* knockout (Alboresi et al. 2010; Peng et al. 2019). It is therefore likely to assume that Lhcb5 is essential for the activity and/or the accumulation of LHCSR1.

There are several possibilities either direct or indirect; i) Lhcb5 is an important regulator for the transcription of *lhcsr1*, ii) Lhcb5, and possibly other gene products regulated by *lhcb5*, is essential for a correct targeting of LHCSR1 towards the chloroplast, iii) Lhcb5 is an essential interaction partner of LHCSR1 and is required for the stable accumulation of LHCSR1 and iv) Lhcb5 is the interaction partner of LHCSR1 and without Lhcb5, LHCSR1 is not able to efficiently quench PSII. In

this stage it is impossible to know which of the previous theories is correct and further research is necessary. One way to identify if *lhcb5* is really involved in the regulation of *lhcsr1*-expression is by creating a new knockout of *lhcb5,* for example with the use of CRISPR-CAS9. Especially interesting would be to knockout *lhcb5* in the *npq4* line expressing LHCSR1, since this would immediately show if there is any relationship between Lhcb5 and LHCSR1 accumulation.

Due to the low accumulation levels of LHCSR1 in the *npq4NoM* mutant, we were unable to ascertain if LHCSR1 is able to interact with LHCII, which is suggested by data from previous research (Pinnola et al. 2015a; Semchonok et al. 2017). In higher plants trimeric LHCII is formed by different combinations of the monomers Lhcb1, Lhcb2 and Lhcb3 (Jackowski et al. 2001), while in *P. patens* the *Lhcbm* genes and an *Lhcb3* ortholog are responsible for the formation of the LHCII trimers (Alboresi et al. 2008). Since LHCSR1 is strictly located in the stroma membranes and the fact that LHCII in *P. patens* is much less confined to the grana regions it is very likely that LHCII is the main interaction partner of LHCSR1 (Pinnola et al. 2015a). In order to narrow down the possibilities of the LHCII-isoforms with which LHCSR1 interacts, we've isolated grana and stroma partitions of *P. patens* by α-DM solubilisation and isolated the light harvesting complexes with a native green gel, see Fig. 4A. The band corresponding to the light harvesting complexes were cut and analysed by mass spectrometry, see Fig. 4B and C. From the mass spectrometry analysis it is clear that LHCSR1 and LHCSR2 together with the PSI antennae are more present in the supernatant fraction which is enriched in the stroma membranes, while most of the LhcbM-isoforms and the PSII monomeric antenna proteins are more abundant in the pellet, enriched in grana stacks. The three LHCII isoforms Lhcb3, LhcbM6 and LhcbM7 are more enriched in the supernatant and thus could be possible interaction candidates for LHCSR1. However, it is unexpected that Lhcb3 is located more in the stroma than in the grana since it was found to hardly attach to PSI in higher plants (Galka et al. 2012) and does not seem to be directly involved in state transitions. On the other hand, Lhcb3 in higher plants is not able to form homotrimers (Jackowski et al. 2001; Caffarri et al. 2004) and

thus could just form heterotrimers with either LhcbM6 and LhcbM7 thereby influencing its location.

Conclusions

By complementing different mutants of *A. thaliana* with LHCSR1 we were able to ascertain that neither Lhcb4 nor Lhcb6 is involved in the quenching activity of LHCSR1. However, due to the low expression levels of LHCSR1 in the absence of *lhcb5* we were unable to determine whether the interaction partner is Lhcb5 and/or LHCII as previously suggested (Pinnola et al. 2015a; Semchonok et al. 2017). Interestingly there seems to be a connection between the absence of the *lhcb5* gene and the expression level of *lhcsr1* in the moss *P. patens* similar to what we observed in the *A. thaliana* mutants (Peng et al. 2019). Moreover, it seems that the NPQ-kinetics of the *lhcb5* knockout mutant in *P. patens* are almost identical to the *lhcsr1* knockout (Alboresi et al. 2010; Peng et al. 2019), thereby suggesting that *lhcb5* is essential for both the accumulation and the interaction with LHCSR1.

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Chapter 4

Growth performance of plants expressing LHCSR1 instead of PSBS

Abstract

A major possibility to improve crop yield lies in the fine-tuning of photosynthesis and the photoprotective mechanisms to ensure minimal energy dissipation while maintaining enough photoprotection. To achieve this, it is essential to completely understand the underlying mechanisms of photosynthesis and photoprotection. Two proteins are essential for the activation of energy quenching (qE), a non-photochemical process protecting plants from sudden exposure to high light, PSBS and LHCSR, found in plants and algae respectively. Previously we've introduced LHCSR1 from *P. patens* into a *A. thaliana npq4*-mutant, a mutant completely devoid of PSBS and thus qE, and found a partial restoration of qE upon transformation with LHCSR1. In this work we compared the growth rate and biomass production of *A. thaliana* Wild Type (WT), the *npq4*-mutant and the *npq4*-mutant transformed with LHCSR1 from *P. patents* in different growth conditions to see whether LHCSR1 gave an advantage in the biomass production. We show that, on the contrary to what is mainly believed, PSBS is not always important in fluctuating light conditions, but that this mainly depends on the timing. Furthermore, LHCSR1 is able to partly replace PSBS in very specific conditions but is never a full substitute.

Introduction

Plants and algae require light to survive, however excess light can be damaging and must therefore be safely dissipated. This process is called Non-Photochemical Quenching and consists of several different components which are active in different time scales. The fastest and most important response is the energy quenching, qE, and dissipates the excess energy as heat. The qE is activated and deactivated in 1-2min and is important for light changes in short timescales, such as canopy movement or clouds passing before the sun. During increased photosynthesis, the Calvin-Benson cycle is not able to keep up with the linear electron flow and the ΔpH over the thylakoid membranes is increased, activating either PSBS or LHCSR, found in plants and algae respectively. Although the precise energy quenching mechanism of both PSBS and LHCSR still remains a mystery, it is clear that it involves an energy transfer between chlorophylls and/or

xanthophylls (Young and Frank 1996; Holt et al. 2005; Bode et al. 2009). Since PSBS does not contain pigments (Dominici et al. 2002) it relies upon an interaction with other antenna proteins, most likely LHCII and Lhcb4 (CP29) (Dall'Osto et al. 2017). LHCSR on the other hand is not only the activator (Liguori et al. 2013; Ballottari et al. 2016), but most probably also the site of quenching due to the fact that it binds Chl a, lutein and violaxanthin and the short lifetime it has in the quenched state (Bonente et al. 2011; Pinnola et al. 2015, 2017).

Since photosynthetic organisms tend to stay on the safe side and dissipate more energy than necessary, it is possible to increase crop yield by decreasing the level of dissipated energy. A previous study showed an increase in crop yield by 15% by modifying the speed of responsiveness of NPQ (Kromdijk et al. 2016). Other studies show that the lack of the NPQ activator, PSBS, increases growth in low light conditions (Khuong et al. 2019). These examples show that relatively simple changes in NPQ can lead to increased growth rates and that even though nature has had millions of years to perfect photosynthesis, it did not do this with the aim to produce the most biomass. Rather plants have evolved to survive and outlast the competition. One strategy to do this, is by harvesting more light than needed, just to ensure that competitors do not receive this light. Furthermore, it is essential for plants to survive specific stresses that may only occur occasionally. Both outlasting competitors and surviving stresses such as drought are essential strategies to survive and thus become the best fitting species, but they are not essential to increase the yield of crops.

Important to get high crop yields is the optimal use of light for the entire field instead of a single individual, meaning that light should not necessarily be converted into heat if it could still be used by a neighbouring plant. Furthermore, most of the abiotic stresses can be limited in a cultivated area, such as watering. Therefore, it might be beneficial for the crop yield if less of the incoming light is converted into heat. Since LHCSR1 from *P. patens* in *A. thaliana* is less active in energy quenching than PSBS, the *npq4* lines expressing LHCSR1 could possibly grow better in certain conditions than WT plants (Dikaios et al. 2019).

In this work we compared the growth rate and biomass production of three *A. thaliana* mutants with different levels of NPQ; Wild Type (WT) having PSBS and thus natural levels of NPQ, the *npq4* mutant which does not contain PSBS and thus lacks most of the NPQ mechanisms and an *npq4* line expressing LHCSR1 from *P. patens* which has a partially restored level of NPQ with different kinetics than observed in WT (Dikaios et al. 2019).

Materials and Methods

Growth conditions

A. thaliana plants (ecotype *Columbia*) were grown in controlled conditions, stable temperature 23°C during the day and 20°C during the night, in a short-day light regime of 8 hours light and 16 hours dark with a light intensity of 150 µmol photons·m^{-2}·s^{-1}. For flowering, plants were put at 12 hours of light and 12 hours of dark.

Fluctuating light system

The system was created using LED tubes on the side for the normal light conditions and 4 high power 3W warm-white LEDs with a 15° focusing lens directly above each plant. Plants were grown in a short-day light regime of 8 hours light and 16 hours dark at different temperatures controlled by a growth chamber. The switching of the high light was controlled by a relay regulated by a programmable Arduino microcontroller (www.arduino.cc). The high-power LEDs were mounted on a large aluminium heat sink actively cooled by fans which were always on. Temperature measurements were performed using an average of 5 individual DS18B20 sensors connected to an Arduino that took a measurement each minute.

Plants were seeded and allowed to germinate for 10 days before begin transplanted into individual clay pots were they were allowed to grow for another week before the experiment was initiated.

Analysis of growth

The growth was monitored by taking pictures of the plants (with a minimal of 10 plants for each genotype and each condition) every 3 or 4 days by counting all the

pixels for each plant using Adobe Photoshop CS6, while using a 1-euro coin as a size reference in the images. After each experiment the fresh weight was determined.

Chlorophyll fluorescence measurements

Four-week-old plants were dark adapted overnight and the Fv/Fm was measured before the start of the experiment. For each experiment 4 plants were measured with a portable chlorophyll fluorometer from Opti-Sciences (www.optisci.com) Before every measurement, plants were exposed to 2s of far-red light to ensure that PSII reaction centres were fully open before the Fv/Fm measurement.

Plants were then exposed to 4 hours 1900µE at 24°C or 4 hours 1300µE at 10°C, the Fv/Fm was monitored every hour. Plants were allowed to recover at 50µE in their respective temperatures and measurements were taken every 15min for the first hour. The last measurement was performed after 24hour of starting the experiment.

Results

We wished to verify if LHCSR1 was capable of partly replacing PSBS in a long-term growth experiment in fluctuating light conditions. We measured the leaf area and biomass production of three different genotypes (WT, *npq4* and *npq4*+LHCSR1) in different fluctuating light conditions.

In order to correctly test the effect of light on the biomass production and thus the capability of these mutants to safely dissipate excess light, we built a special system capable of providing normal light at (100-150µE) and high light (up to 1200µE) without introducing any large temperature fluctuations to the plants, maximum of 2.5°C increase after 1 hour of 1200µE, see Fig. 1.

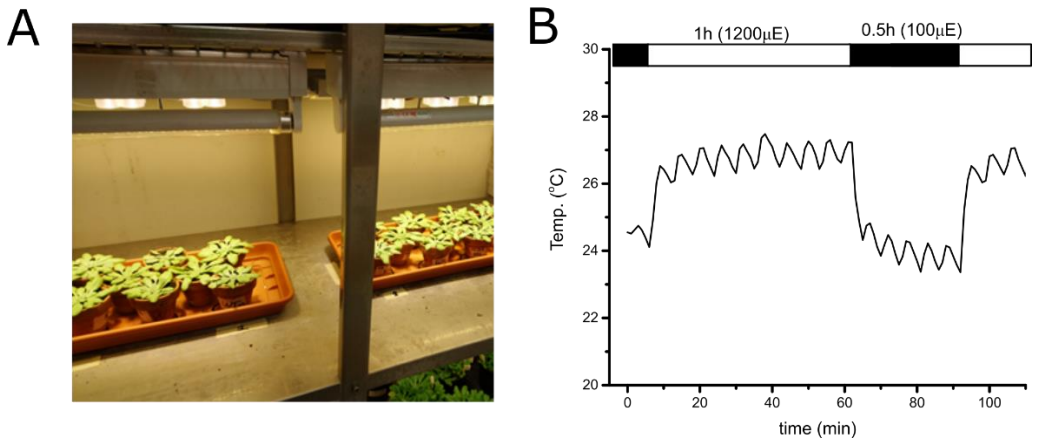

Figure 1 Overview of the fluctuating light system and the temperature measured

The fluctuating light system is capable of switching between normal light (100-150µE) and high light (600-1200µE) in seconds without introducing too much heat to the plants. A) overview of the system and an experiment in progress with the high-power LEDs on. B) Overview of the temperature that the plants experience during the 1h 1200µE/0.5h 100µE experiment.

The system was created using LED tubes on the side for the normal light conditions and 4 high power 3W warm-white LEDs with a 15° focusing lens directly above each plant, see Fig. 1A. The high-power LEDs were mounted on a large aluminium heat sink actively cooled by air. This enabled us to only determine the effect of light and not of any variations introduced by temperature, something which is sometimes overlooked in growth experiments.

Effect of fluctuating light on growth

To test whether fluctuating high light could influence the biomass production we grew plants in a controlled temperature with 1-hour 1000µE and half an hour of 100µE. As a control we grew plants at continuous light in the same environment at 100µE. To see whether this had any effect on the growth we determined the total leaf area every 3 or 4 days and the biomass production in the form of the fresh weight at the end of each experiment, see Fig. 2.

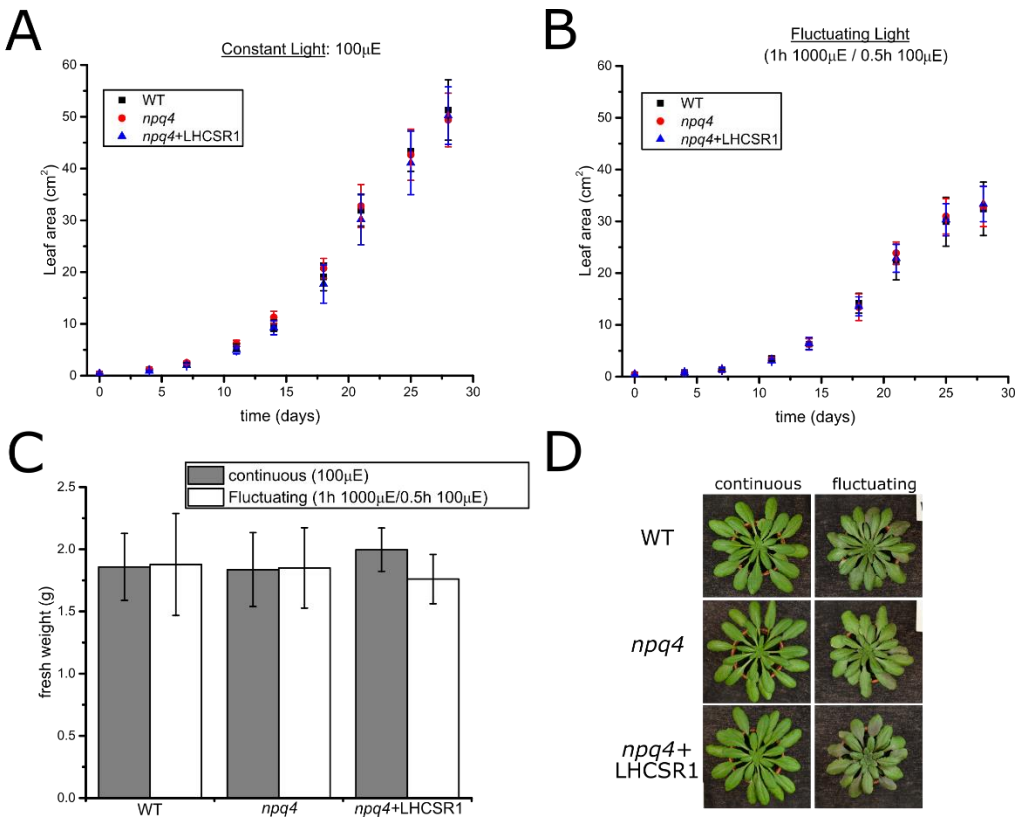

Figure 2 Growth in continuous and fluctuating light in *A. thaliana*

Growth of the different mutants (WT, npq4 and npq4+LHCSR1) in continuous (100µE) and fluctuating light (1h 1000µE / 0.5h 100µE). A) Leaf area of the three mutants during the continuous light. B) Leaf area in fluctuating light. C) Fresh weight of both experiments measured at the end. D) Pictures of plants grown in continuous and fluctuating light at the end of the experiment.

The leaf area of the plants grown in the constant light are approximately 1.5 times bigger than that of the plants gown in the fluctuating light, Fig. 2A and B. However, the fresh weight of both the experiments are very similar and there is no statistically significant difference between the genotypes nor between the different growth conditions, Fig. 2C. Plants grown in fluctuating conditions show a more cropped phenotype in comparison to the plants grown in continuous light, they furthermore show some indications of stress as can be seen from the purple spots, see Fig. 2D.

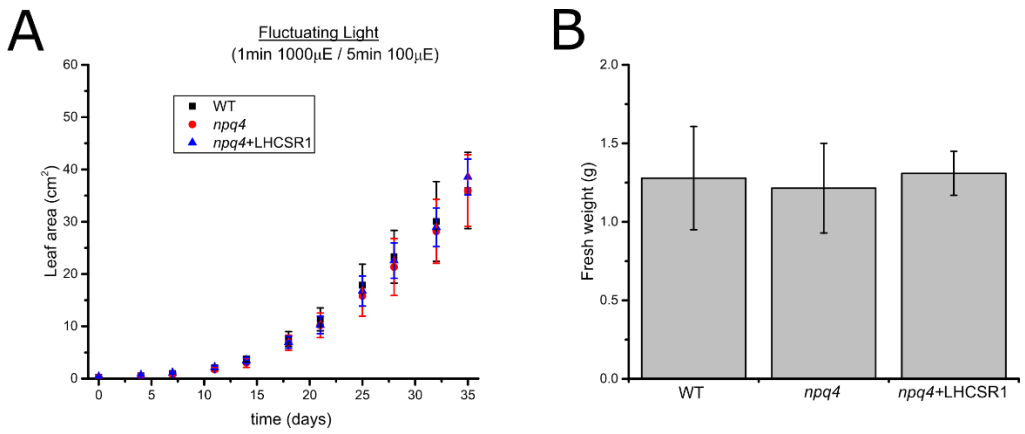

Figure 3 Growth in fluctuating light (1min 1000µE / 5min 100µE)

Growth of the different mutants in fluctuating light (1min 1000µE / 5min 100µE). A) Leaf area of the three mutants during the fluctuating light. B) Fresh weight of the plants grown in fluctuating light.

Due to the lack of difference in fluctuating light conditions between WT and the *npq4* mutant we thought that the fluctuating light periods were too long. However, in another paper not necessarily focused on the difference in growth between WT and *npq4* mutants it seemed that a difference could be observed between these two mutants with a light period of 1min 600µE / 5min 60µE (Grieco et al. 2012). Instead of using 600µE and 60µE, we used 1000µE and 100µE with the idea that this should increase the level of stress experience by the plants. However, no significant differences were observed between the different genotypes, neither in the leaf area nor in the fresh weight at the end of the experiment, see Fig 3A and B.

To check whether 1 minute is not enough to induce stress, this was extended to 5min of high light, see Fig. 4. In this condition there was a significant difference between WT and *npq4*/*npq4*+LHCSR1 both in leaf area and fresh weight. Indicating that WT was able to grow better in these stressing conditions than the *npq4* mutant and the *npq4* transformed line. However, no difference between *npq4* and *npq4* expressing LHCSR1 was observed.

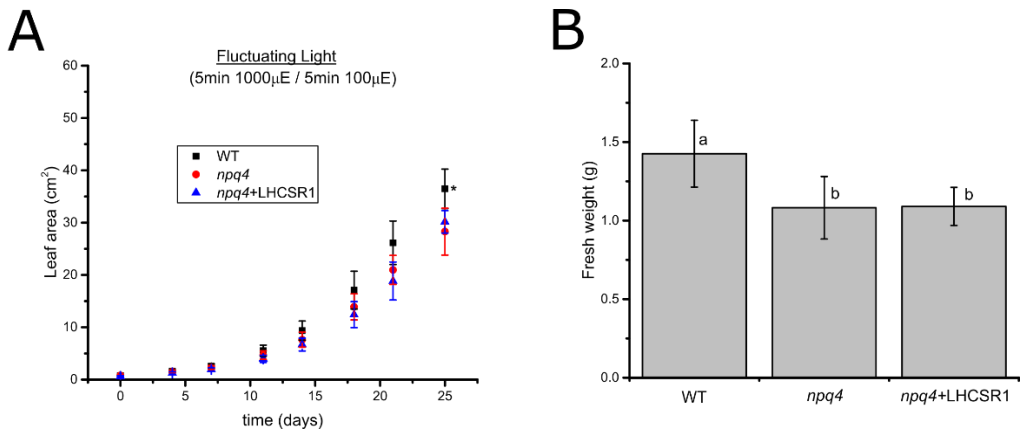

Figure 4 Growth in fluctuating light (5min 1000µE / 5min 100µE)

Growth of the different mutants in fluctuating light (5min 1000µE / 5min 100µE). Significant difference (ANOVA $p<0.05$) indicated by a star or by different letters. A) Leaf area of the three mutants during the fluctuating light. B) Fresh weight of the plants grown in fluctuating light.

Effect of fluctuating light on growth with additional stress

Since plants are rarely exposed only to high light, we also wished to test the effect on the biomass production with the addition of another abiotic stress. Especially since we were not able to see any positive effect induced by the transformation of LHCSR1. Since plants when grown during spring are exposed to low temperatures and high light (Poorter et al. 2016), we performed the same experiment at 4°C with the 5min fluctuating light interval, see Fig. 5. While plants did grow in the 100µE continuous light at 4°C, albeit much slower than in normal conditions, the fluctuating light treatment turned out to be too extreme and all the plants were dead after 7 days of starting the experiment. However, WT did survive longer than both *npq4* and *npq4*+LHCSR1.

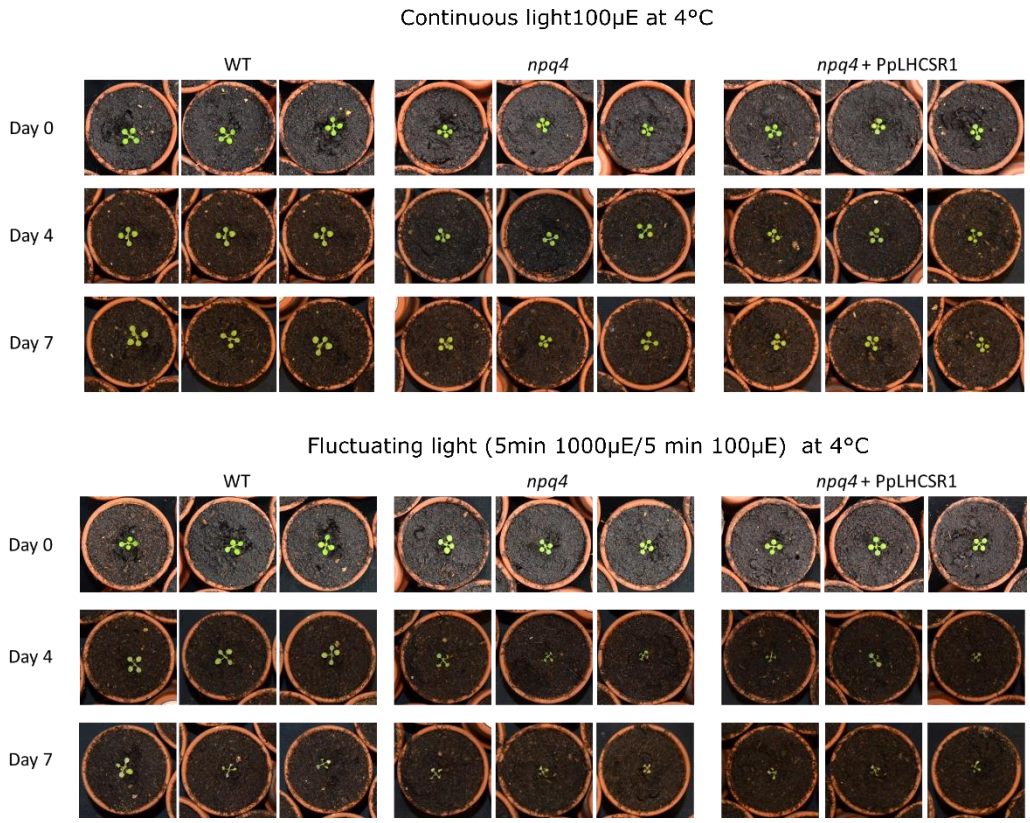

Figure 5 Growth at 4°C in continuous (100µE) and fluctuating light (5min 1000µE / 5min 100µE)

Growth of the different mutants in fluctuating light (5min 1000µE / 5min 100µE) at 4°C. Three out of the 11 were randomly chosen and photographed.

To ensure that plants would not die, we increased the temperature to 10°C and reduced the intensity of the high light to 600µE, while keeping the same fluctuating light interval. Plants did grow much slower than at 24°C even in continuous light, see Fig 6A, but no significant difference between the genotypes was observed. In the fluctuating light conditions however, a difference between WT and *npq4/npq4*+LHCSR1 could be observed ($p < 0.05$), where WT grows much better than the mutants, see Fig. 6. Especially interesting is the difference between WT in continuous light and fluctuating light. Where in the other two genotypes there is a reduction in the fresh weight for plants grown in fluctuating light, WT actually produced more biomass in these conditions ($p < 0.05$), while this was not the case in the other experiments. However, the leaf area of the WT plants in continuous light is larger than that of the plants grown in fluctuating light. Indicating that there is a discrepancy between fresh weight and the leaf area.

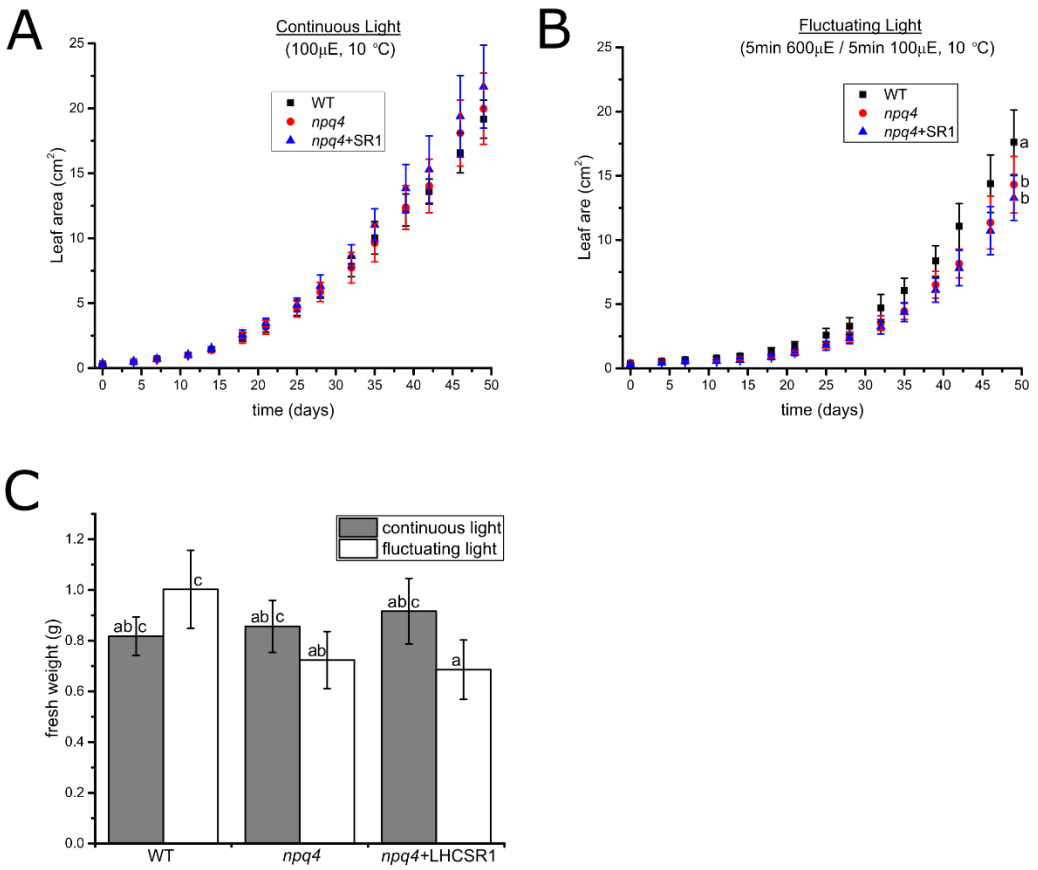

Figure 6 Growth at 10°C in continuous and fluctuating light

Growth of the different mutants (WT, npq4 and npq4+LHCSR1) in continuous (100µE) and fluctuating light 5min 600µE / 5min 100µE) at 10°C. A) Leaf area of the three mutants during the continuous light. B) Leaf area in fluctuating light. C) Fresh weight of both experiments measured at the end. Statistically significant differences are indicated by different letters (Anova, $p < 0.05$).

Since plants did not seem to be too stressed at 10°C with 600µE and we wished to increase the difference between the genotypes by increasing the high light intensity to 1000µE while keeping the rest of the parameters the same (temperature, timing). Both the leaf area and the fresh weight of the WT showed a significant difference with both *npq4* and *npq4*+LHCSR1 ($p < 0.05$) as was the case at 24°C with the 5min fluctuation (Fig. 4). However, there was now also a significant difference between *npq4* and *npq4*+LHCSR1 both in the leaf area and the fresh weight, where the transformed *npq4* mutants had a bigger leaf area and produced more biomass, see Fig. 7.

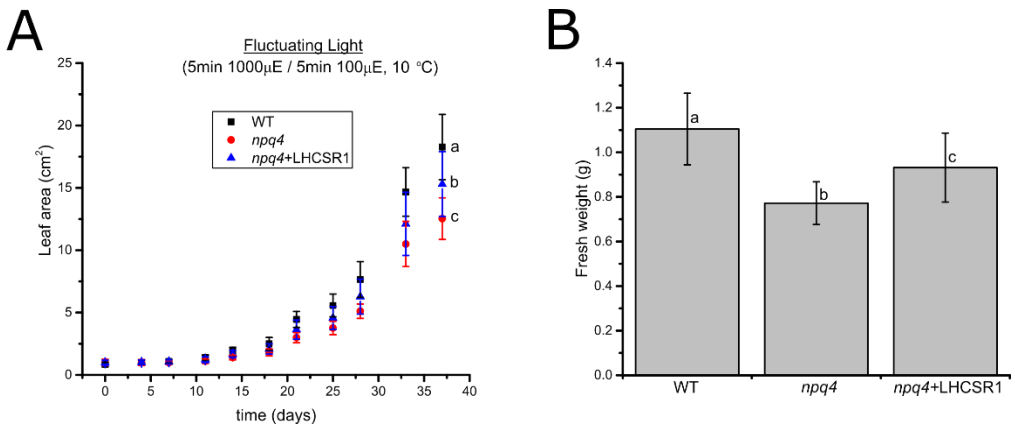

Figure 7 Growth at 10°C in fluctuating light (5 min 1000µE / 5 min 100µE)

Growth of the different mutants (WT, npq4 and npq4+LHCSR1) in fluctuating light (5min 1000µE / 0.5h 100µE) at 10°C. A) Leaf area of the plants grown in fluctuating light. B) Fresh weight of the plants grown in fluctuating light. Statistically significant differences are indicated by different letters (Anova, $p < 0.05$).

Since there was no significant difference observed between *npq4* and *npq4*+LHCSR1 in fluctuating light at 24°C, but there was a difference at 10°C, we wished to verify if the additional abiotic stress of the cold had an influence on the repair of PSII. To measure this, we followed the Fv'/Fm', which gives an indication for the amount of intact PSII-core complexes during high light stress and the recovery, see Fig. 8. We measured at both 24°C and 10°C and plants were exposed to 4 hours of high light (1900µE at 24°C and 1300µE at 10°C) induce PSII-damage. Then plants were allowed to recover at their respective temperatures at 50µE. At 24°C the damage to PSII is less pronounced in the WT during the high light stress. During the recovery WT shows a very fast increase in the Fv'/Fm' in the first hour of the recovery which is most likely caused by the deactivation activity of PSBS. However, it is clear that after 24h, WT shows a better recovery than both *npq4* and *npq4*+LHCSR1. The kinetics of both *npq4* and *npq4*+LHCSR1 are very similar at 24°C, but interestingly enough become different when the experiment is repeated at 10°C, where *npq4*+LHCSR1 shows a faster recovery in the first hour at 50µE, right between WT and *npq4* as was observed in the fresh weight and leaf area during the growth in the fluctuating light at 10°C. However, after 24h, both *npq4* and *npq4*+LHCSR1 have the same Fv'/Fm', while the Fv'/Fm' of WT remains higher during the whole recovery period.

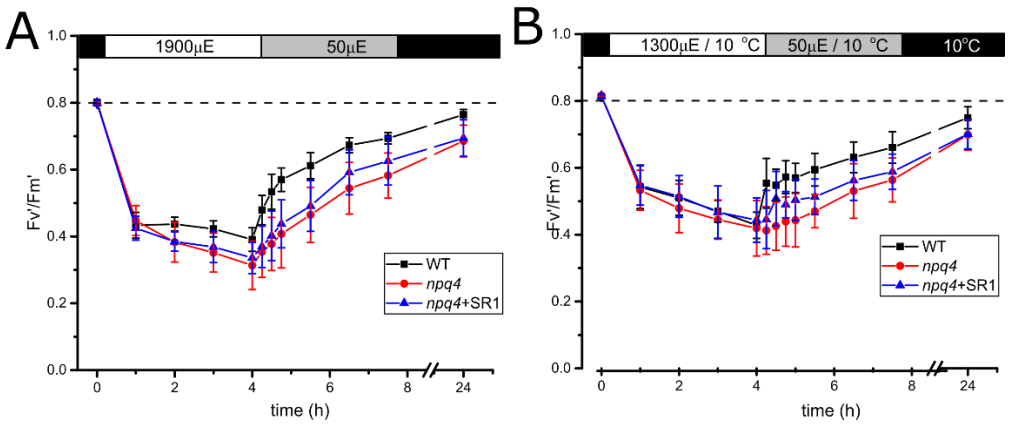

Figure 8 Fv'/Fm' in time during high light stress and recovery

The Fv'/Fm' was measured for the different mutants. To damage PSII-core complexes, the plants were exposed to high light for 4 hours and then allowed to recover at 50µE for another 4 hours. The final point was measured after 24hours after starting the experiment. A) Fv'/Fm' of plants exposed to 1900µE at 24°C and their recovery at 24°C. B) Fv'/Fm' of plants exposed to 1300µE at 10°C before they were allowed to recover at 10°C.

Discussion

Plants can adapt to different light conditions and when grown in continuous high light, even the absence of PSBS does not lead to a reduction in biomass in comparison to WT (Golan et al. 2006), which is attributed to a structural change in the photosynthetic machinery: increased photosynthetic and decreased light-harvesting capacity. However, plants do not experience continuous light in nature and therefore constantly balance between light-harvesting and energy quenching. Since photosynthetic organisms tend to stay on the safe side and dissipate more energy than necessary, it is theoretically possible to increase crop yield by decreasing the total level of dissipated energy, as previously shown (Kromdijk et al. 2016)

Previously we've successfully transformed *A. thaliana npq4* plants with LHCSR1 from *P. patens*, the transformed plants show a recovery of NPQ, dependent on the accumulation of zeaxanthin (Dikaios et al. 2019). However, it was not clear whether these plants, having a decreased level of NPQ, could increase the crop yield in certain conditions or if they provide an adequate protection to replace PSBS. The aim of this work was to discover if LHCSR1 can replace PSBS and/or improve the biomass production. We therefore built a high light system, capable of providing

fluctuating light of up to 1200µE without heating up the plants to ensure that plants were not subjected to any other abiotic stress than light, see Fig. 1A and B.

We first tried to find a fluctuating light period that would yield a difference between WT and *npq4*, however this proved to be more difficult than previously anticipated. The first light regime, one-hour high light (1000µE) and half an hour normal light (100µE), did not show any significant differences between the different genotypes, not in the leaf area nor in the biomass production. However, the leaf area of the plants grown in the constant light were approximately 1.5 times bigger than that of the plants gown in the fluctuating light, while the fresh weight of both conditions were similar, Fig. 2A, B and C. This indicates that the leaf area is not always a good measure for the estimation of the biomass production. The plants grown in the fluctuating light had more cropped rosettes and thicker leaves than those grown in continuous light, see Fig. 2D. Furthermore, the plants grown in fluctuating light showed some purple colorization, indicating the production of anthocyanins which is normally induced by abiotic and/or biotic stress (Ougham et al. 2005). Although it is impossible to know why there was no difference between WT and *npq4* plants, a theory is that the plants, both WT and *npq4*, are adapting the size of their antenna complexes to the longer high light period and bridge the low light period, where they are able to repair any damage that might have occurred to PSII during the lower light periods. Furthermore, there is also another photoprotection mechanism independent of PSBS that operates in the longer time regimes, the chloroplast movement (qM), which might reduce the overall damage (Dall'Osto et al. 2014).

To avoid any effect of long-term high light adaptation or chloroplast movement, we grew plants at 1 min 1000 µE and 5 min 100µE. This light period was used in another paper, focused on mutants unable to perform state-transitions and not on the level of inducible NPQ, where a difference in growth could be observed between WT and *npq4* plants with a light period of 1min 600µE / 5min 60µE (Grieco et al. 2012). Since we are looking at abiotic stress induced by high light, the increased light intensities (1000µE instead of 600µE and 100µE instead of 60µE) used in our experiment should have a stronger effect and increase the difference between WT and *npq4*. However, in this experiment we did not observe a significant difference

between the different lines, not in the fresh weight nor in the leaf area, see Fig. 3A and B. Our theory was that the one-minute high light might be too short to induce any significant damage, we therefore increased the high light period to 5 min and in this case, there was a significant difference between WT and *npq4*, see Fig. 4. WT produced approximately 25% more biomass than either *npq4* or *npq4*+LHCSR1, yet there was no difference between *npq4* and *npq4*+LHCSR1.

However, there was a difference in growth between *npq4* and *npq4*+LHCSR1 if the plants were grown in the same fluctuating light period at 10°C, see Fig. 7. From these results it can be concluded that LHCSR1 does not really contribute to photoprotection in fluctuating high light alone but does seem to make a difference if another abiotic stress is added, in this case a lower temperature. This could have two possible explanations, either the additional abiotic stress is becoming too much for the plants and any form of photoprotection is helpful or LHCSR1 becomes more important during lower temperatures.

There are two isoforms of LHCSR found in *P. patens*, LHCSR1 and LHCSR2, these two isoforms have a 91% amino-acid sequence identity indicating a similar biochemical activity (Alboresi et al. 2010). Of the two, LHCSR1 is expressed in higher quantities and therefore responsible for most of the NPQ found in *P. patens* (Alboresi et al. 2010). However, LHCSR1 and LHCSR2 were found to be differentially regulated, where LHCSR1 was overexpressed during high light conditions, LHCSR2 was upregulated during exposure to low temperatures, suggesting a role for LHCSR2 in photoprotection during low temperatures (Gerotto et al. 2011). However, when LHCSR1 is knocked out in *P. patens* there is a clear increase in damaged PSII during lower temperatures, something which is not observed when PSBS is absent (Gerotto et al. 2011). This would suggest a role for photoprotection in lower temperatures for LHCSR1 as well, which corresponds to the results we found where *npq4* plants expressing LHCSR1 grew better in high light at lower temperatures than the *npq4* plants, see Fig 7.

This is confirmed when we look at the Fv/Fm traces of the plants at 24°C and 10°C, see Fig. 8. The Fv/Fm gives a measure for the amount of PSII reaction centres that can perform photochemistry. For one, the Fv/Fm trace of WT shows less decline

than *npq4* and *npq4*+LHCSR1, which can be attributed to the photoprotection mechanism of energy quenching by PSBS, leading to less damage to the reaction centres, but there is also a very fast initial recovery visible in the first hour at 50µE. This initial fast recovery is mostly caused by the relaxation of the NPQ mechanisms and not only by the turnover of repaired PSII reaction centres. The recovery of *npq4* and *npq4*+LHCSR1 at 24°C is very similar, but in the cold *npq4*+LHCSR1 shows a different recovery from *npq4*, especially in the first hour. This is interesting, because this initial rise was not visible at 24°C, which would lead to the conclusion that LHCSR1 was mostly active in NPQ, and thereby reducing the Fv/Fm, at 10°C and not so much at 24°C. However, previously NPQ measurements of *npq4*+LHCSR1 were always performed at room temperature and NPQ was induced in these conditions as well (Dikaios et al. 2019). Also, LHCSR1 did not provide any advantage in biomass production at 10°C in lower fluctuating light conditions (600µE), see Fig. 6. Furthermore, control plants grown at 10°C in continuous light did not show any difference at all. Therefore, the increased biomass production of plants expressing LHCSR1 cannot be solely attributed to the lower temperatures but is most probably caused by the combination of the two extreme stresses, high light (1000µE) and lower temperatures (10°C). This becomes clear when the temperature is lowered to 4°C and the high light is kept at 1000µE, see Fig. 5, in these conditions all the plants were dead after 7 days, but it is clear that WT is able to survive the stress better than both *npq4* and *npq4*+LHCSR1. Therefore, it seems that it is not necessarily the cold where LHCSR1 gives an advantage in protection, but most likely the combination of two stresses where LHCSR1 was able to partly counteract photodamage, although not as efficient as PSBS.

Photosystem II is constantly being repaired as it is very sensitive to photo-inhibition, the process of photo-inhibition and repair occur simultaneously. Leading to a relatively simple interpretation, where active and inactive PSII must be in balance for optimal growth and where the level of inactive PSII increases when the rate of repair is lower than the rate of photo-inactivation (Nishiyama and Murata 2014). Previously it has been shown that the rate of damage to PSII is proportional with the light intensity (Tyystjärvi and Aro 1996), however the rate of PSII repair already reaches a maximum at low light intensities (Allakhverdiev and Murata

2004). Therefore, in high light conditions, the rate of photo-inhibition is high, and the repair mechanism is not able to keep up, leading to a disbalance in active vs inactive PSII and therefore photo-inhibition. In these conditions, plants and algae decrease the amount of light 'received' by PSII by dissipating a large part of the energy. However, the addition of other abiotic stress such as cold, heat or drought can inhibit the repair mechanism (Barrs 1971; Greer et al. 1986; Wingler et al. 2000) and therefore further increase the necessity of energy dissipation.

Conclusion

In order to improve the yield of crops it will be necessary to fine-tune the photoprotective mechanisms to ensure minimal energy dissipation while maintaining enough photoprotection. This will lead to different levels and probably different types of photoprotection, depending on the growth conditions. Plants in greenhouses are relatively well protected against high light, drought and cold, while plants in the field might need to be more drought resistant and cope with fluctuating high light. In this work we investigated whether LHCSR1 could increase biomass production or replace PSBS in specific conditions. This is not the case, as LHCSR1 was only able to increase performance of *npq4* transformed lines in very specific conditions, but was never able to compete with PSBS found in WT. LHCSR1 did seem to be more important in conditions where an additional abiotic stress (cold) was added in addition to the fluctuating high light, but it is not yet clear whether the small contribution of NPQ was more important than a real protection against lower temperatures. However, should LHCSR1 be expressed in WT plants, this might increase the total level of protection even further and might increase biomass production or survival rate in extreme conditions.

Literature

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Chapter 5

LHCI mediates excitation-energy transfer from LHCII to photosystem I in *Arabidopsis*

Christo Schiphorst[1], Luuk Achterberg[2], Rodrigo Gómez[1], Rob Koehorst[2,3], Roberto Bassi[1], Herbert van Amerongen[2,3], Luca Dall'Osto[1], Emilie Wientjes[2*]

[1]Dipartimento di Biotecnologie, Università di Verona, Strada Le Grazie 15, 37134 Verona, Italy

[2]Laboratory of Biophysics, Wageningen University, P.O. Box 8128, 6700 ET Wageningen, The Netherlands

[3]MicroSpectroscopy Research Facility, Wageningen University, P.O. Box 8128, 6700 ET Wageningen, The Netherlands

*Corresponding author: Emilie.Wientjes@wur.nl

This article has been submitted to Plant Physiology

Abstract

Photosynthesis powers nearly all life on Earth. Light absorbed by photosystems drives the conversion of water and carbon dioxide into sugars. In plants, photosystem I (PSI) and photosystem II (PSII) work in series to drive the electron transport from water to $NADP^+$. As both photosystems largely work in series, a balanced excitation pressure is required for optimal photosynthetic performance. Both photosystems are composed of a core and a light-harvesting complex, LHCI for PSI and LHCII for PSII. When the light conditions favor the excitation of one photosystem over the other, a mobile pool of trimeric LHCII moves between photosystems thus tuning their antenna cross-section in a process called state transitions. When PSII is over-excited multiple LHCIIs can associate with PSI. A trimeric LHCII binds to PSI at the PsaH/L/O site to form a well characterized PSI-LHCI-LHCII supercomplex. The binding site(s) of the other "additional" LHCII is still unclear, although a mediating role for LHCI has been proposed. In this work we measured the PSI antenna size and trapping kinetics of photosynthetic membranes from *A. thaliana* plants. Membranes from WT plants were compared to the *ΔLhca* mutant that completely lacks the LHCI antenna. The results show that "additional" LHCII complexes can transfer energy directly to the PSI core in the absence of LHCI. However, the transfer is at least four times faster, and therefore more efficient, when LHCI is present. The data indicates that LHCI has specific LHCII binding sites which enhance the LHCII to PSI energy transfer.

Introduction

In oxygenic photosynthesis photosystem I (PSI) and photosystem II (PSII) work in series to oxidize water and reduce $NADP^+$. Both photosystems are composed of a core complex, which comprises the reaction center and the electron transport chain, and an outer light-harvesting (antenna) system (Blankenship, 2014; Croce and van Amerongen, 2020). In higher plants the antenna of PSI (LHCI) is composed of 4 Lhca complexes, called Lhca1-4 (Knoetzel et al., 1992; Jansson et al., 1997; Jansson, 1999; Croce et al., 2002; Ben-Shem et al., 2003; Wientjes and Croce, 2011). The light-harvesting cross section of the PSII core is enlarged by the monomeric CP24 (Lhcb6), CP26 (Lhcb5) and CP29 (Lhcb4) and trimeric LHCII

complexes (combinations of Lhcb1-3) (Jansson et al., 1997; Jansson, 1999). A special pool of LHCII trimers can associate with either PSI or PSII based on the light conditions (Allen et al., 1981; Bassi et al., 1988). PSI and PSII are both embedded in the thylakoid membrane, but their distribution is very heterogeneous. PSII is mainly found in the stacked grana membranes, while PSI is located in the interconnecting unstacked stroma lamellae membranes (Andersson and Anderson, 1980; Dekker and Boekema, 2005).

As PSI and PSII work in series, a balanced excitation between the two photosystems is required for optimal photosynthetic efficiency. However, PSI and PSII have different absorption spectra and during the day the light spectral composition can change depending on the time of the day and the position of the leaves in the canopy (Croce and van Amerongen, 2014; Johnson and Wientjes, 2020). At twilight, when the sun sets below the horizon, the short-wavelength content of ambient light becomes enriched due to the increased amount of ozone absorption (Spitschan et al., 2016). Blue light of 460-490 nm is more readily absorbed by PSII, thus resulting in over-excitation of this photosystem. Instead, below a canopy the blue and red part of the sun light is absorbed by upper leaves and the spectrum is enriched in green and far-red light. The red forms of PSI absorb the light above 700 nm, thus resulting in over-excitation of this photosystem (Coombe, 1957; Hogewoning et al., 2012; Johnson and Wientjes, 2020).

State transitions are the well-known acclimation mechanism which rebalance the excitation pressure on the photosystems by relocating LHCII between PSI and PSII (Allen, 2003; Rochaix, 2014; Goldschmidt-Clermont and Bassi, 2015). When PSII is over-excited the Stn7 kinase phosphorylates the Lhcb2 isoform of a mobile pool of $Lhcb1_2Lhcb2_1$ LHCII trimers. The phosphorylated LHCII complex moves to PSI (State 2), where it binds to the PsaL/H/O site of the PSI core and forms a digitonin resistant PSI-LHCI-LHCII complex (Bellafiore et al., 2005; Kouril et al., 2005; Galka et al., 2012; Crepin and Caffarri, 2015; Longoni et al., 2015; Pan et al., 2018). Instead, when PSI receives too much excitation energy, the LHCII is dephosphorylated by the TAP38/PPH1 phosphatase and moves to PSII (State 1) (Pribil et al., 2010; Shapiguzov et al., 2010). Although it has been assumed since a

long time that state transitions increase the photosynthetic efficiency in higher plants, proof for such a relationship was only presented recently (Taylor et al., 2019).

The nomenclature, State 1 and State 2, might give the impression that there are only two absolute states, however this is not the case. While State 1 can be described as the condition where LHCII is not phosphorylated and PSI-LHCI-LHCII complexes are absent, this is not the same for State 2. Under most of the light regimes usually experienced by plants, part of the "extra" LHCII pool is phosphorylated and enlarges the PSI antenna in the PSI-LHCI-LHCII supercomplex, thus resulting in a "partial" State 2 (Tikkanen et al., 2008; Wientjes et al., 2013). Yet, illuminating the leaves with light that specifically over-excites PSII e.g. ~470 nm or ~650 nm light (Hogewoning et al., 2012), leads to a more "extreme" State 2.

Besides the association of one LHCII trimer with PSI in state 2, several reports indicate that unphosphorylated (digitonin sensitive) LHCII can function as PSI antenna in State 1 (Benson et al., 2015; Bressan et al., 2018; Bos et al., 2019) and that more than one LHCII complex can increase the PSI absorption cross section (Bassi and Simpson, 1987; Andreasson and Albertsson, 1993; Jansson et al., 1997; Bell et al., 2015; Benson et al., 2015; Grieco et al., 2015; Bos et al., 2017; Bressan et al., 2018; Bos et al., 2019; Chukhutsina et al., 2020). As mutant plants devoid of specific Lhcas also show impaired state-transitions, it has been suggested that these "additional" non-PsaH/L/O binding LHCII trimers transfer energy to the LHCI-site of PSI-LHCI (Benson et al., 2015). However, a subsequent study on *ΔLhca A. thaliana* plants, that lack all four Lhca antennas, showed that the thylakoid architecture of these plants is significantly modified with respect to the wild type. It was concluded that the altered thylakoid organization makes the chlorophyll fluorescence analysis of state transitions problematic (Bressan et al., 2018). As such, it is at present not clear if the "additional" LHCII trimers indeed transfer their excitation energy to the LHCI site of PSI-LHCI, let alone how fast and efficient the energy transfer is.

In this work we investigate the role of LHCI in mediating the energy transfer from LHCII to PSI in the stroma lamellae membranes. To this end, we

compared the PSI antenna size and excitation energy transfer and trapping in State 1 *vs.* State 2 membranes from wild type and *ΔLhca A. thaliana* plants.

Materials and Methods

Plant material and growth conditions – WT plants of *A. thaliana* (Col-0), mutant *ΔLhca* (Bressan et al., 2018) and double mutant *stn7ΔLhca*, obtained by crossing the *stn7* mutant (Bellafiore et al., 2005) with the *ΔLhca* mutant (Bressan et al., 2018), were grown for 6 weeks at 150 μmol photons m^{-2} s^{-1}, 8 hours of daylight (OSRAM halogen HQI-T 250W and/or OSRAM lumilux cool white L58W) and 23/20°C day/night at a humidity of 70%.

Thylakoid and stroma lamellae isolation – State 1 and state 2 were induced as in (Bressan et al., 2018) with slight modifications; leaves from overnight dark-adapted plants were placed on wet paper for 45 min with either PSI light (a combination of far-red LEDs with a peak at 730nm and 850nm) or PSII light (30W warm white fluorescent lamps filtered with Lee 105 orange filters). Thylakoids were isolated as previously described (Berthold et al., 1981). Leaves treated with state 1 or state 2 light were directly homogenized using ice-cold buffer B1 (20 mM tricine-KOH pH 7.8, 0.4 M NaCl, 2 mM $MgCl_2$, 0.5% milk powder) and filtered with a nylon mesh before centrifugation at 1500g, 4°C for 12 min. The pellet was washed in buffer B2 (20 mM tricine-KOH pH 7.8, 0.15 M NaCl, 5 mM $MgCl_2$) before centrifugation at 4000g, 4°C for 12 min. The pellet was resuspended in buffer B3 (20 mM HEPES-KOH pH 7.5, 15 mM NaCl, 5 mM $MgCl_2$) and centrifuged at 6000g, 4°C for 12 min. If thylakoids were stored, they were resuspended in buffer B4 (20 mM HEPES-KOH pH 7.5, 0.4 M Sorbitol, 15 mM NaCl, 5 mM $MgCl_2$) and immediately frozen in liquid nitrogen before storage at -80°C. Buffer B1 to B3 were supplemented, right before grinding or resuspending, with protease inhibitors (0.1 mM benzamidine, 0.1 mM PMSF, 0.5 mM aminocaproic acid) and freshly prepared 10 mM sodium fluoride. Stroma lamellae isolation was performed as previously described (Barbato et al., 2000), from freshly prepared thylakoids in buffer B3. Solubilized thylakoids were 7 times diluted with buffer B3 before the centrifugation steps.

PSI core isolation – A. thaliana ch1 plants (ordered from TAIR database: CS126), which lack chlorophyll *b* and accumulate the PSI core without LHCI associated (Havaux et al., 2007), were grown as described above and the thylakoids were isolated. Thylakoids at a chlorophyll concentration of 1 mg/ml were dissolved with an equal volume of 1.2% n-Dodecyl β-D-maltoside. After 5 minutes incubation on ice the mixture was centrifuged for 1 min at 13000 g and the supernatant was loaded on a 0.1 – 1 M sucrose density gradient as described in (Wientjes et al., 2009). The bands were harvested with a syringe and the PSI core band (monomeric and oligomeric state) were identified based on position in the gradient, chlorophyll content and absorption spectra.

Normalization of absorption spectra – The absorption spectra of LHCII, PSI-LHCI (Hogewoning et al., 2012) and PSI core were normalized to the same number of chlorophylls taking into account the chlorophyll a/b ratios of 1.3 (LHCII), 9.7 (PSI-LHCI) and ∞ (PSI-core) and an oscillator strength for chlorophyll *b* of 0.7 times chlorophyll *a* in the region from 630 – 750 nm (Sauer et al., 1966).

Pigment Analysis – The pigment composition of the different states were analysed by fitting the acetone extract spectrum with the spectra of the individual pigments as described (Croce et al., 2002).

PSI antenna size measurements – P700 measurements were performed as described before (Benson et al., 2015) on a Walz DUAL-PAM-100 in the dual wavelength mode (830 and 875nm) using a concentration of 50 µg chlorophylls per mL in the measuring buffer (0.4 M sorbitol, 15 mM NaCl, 5 mM $MgCl_2$, 10 mM HEPES pH7.5 buffered KOH, 50 µM DCMU, 100 µM methylviologen, 500 µM sodium ascorbate). The following protocol was used for 5 cycles on every sample: 5 s dark, 10 s red light (635 nm) at 6 µmol photons m^{-2} s^{-1} and 30 s dark recovery. Traces were normalized between the minimum (beginning of each cycle) and the maximum and fitted with an exponential function to determine the $t_{1/2}$ for the antenna size calculations. PSI antenna sizes are expressed as a percentage of the WT State 1 value.

Polyacrylamide gel electrophorese – For the large pore – blue native gel the samples and gels were prepared as described (Jarvi et al., 2011), and solubilisation

of samples was carried out with 0.1% α-DM and 0.5% digitonin final concentration as described previously (Galka et al., 2012). In total, 40 µg of chlorophylls were loaded in a medium-sized gel (16 cm height). For the SDS-PAGE, a modified Laemmli gel was used as described in (Laemmli, 1970; Ballottari et al., 2004).

Streak-camera measurements – Time-resolved fluorescence measurements were performed with a streak-camera system as described previously (van Oort et al., 2009). The sample was measured in a 1 cm×1 cm cuvette at a chlorophyll concentration of 20 µg/ml and continuously stirred during the measurement. To excite the sample, pulsed laser light with a repetition rate of 3.8 MHz, a wavelength of 400 nm and an intensity of ~50 µW, was focused in a spot with a diameter of ~100 µm. Time-windows of 2 ns and 800 ps were used for the measurements. The collected streak images were corrected for background signal and for spatial variation of detection sensitivity. The corrected datasets were globally analyzed using Glotaran and described with decay-associated spectra (Mullen and van Stokkum, 2007; Snellenburg et al., 2012).

Results

State transitions and changes of the PSI antenna size – In order to investigate the role of LHCI in state transitions, we analyzed thylakoids purified from detached leaves of WT, *ΔLhca* and *Stn7ΔLhca* plants after illumination of 45 minutes with far-red light (State 1) or orange light (State 2), in order to over-excite respectively PSI and PSII. Thylakoids from these plants were isolated and the chlorophyll *a/b* ratio was measured (Table 1). The chlorophyll *a/b* ratio of the WT and mutant plants were not affected by the 45-minute light treatment, indicating that the PSI/PSII ratio and LHCII/PSII ratio did not change during the treatment. The thylakoids were solubilized with digitonin and the supernatant was analyzed with native PAGE. Digitonin is a mild detergent, which solubilizes the unstacked parts of the thylakoid membrane and preserves the binding interaction between the P-Lhcb2_1-Lhcb1_2 trimer and the PsaH/L/O side of the PSI core (Zhang and Scheller, 2004; Kouril et al., 2005; Galka et al., 2012; Pan et al., 2018). After illumination with far-red light the PSI-LHCI-LHCII complex is absent in WT plants, likewise the PSI_{core}-LHCII complex is absent in *ΔLhca* plants. Instead, illumination with orange light resulted in the association of LHCII to about 50% of the PSI-LHCI complexes in WT plants. Almost all PSI was found in the PSI_{core}-LHCII complex of *ΔLhca* plants, in

agreement with previous results (Bressan et al., 2016). As expected, the PSI_{core}-LHCII complex was not observed in *Stn7ΔLhca* plants, which lack the LHCII kinase required for the phosphorylation of Lhcb2-$Lhcb1_2$. It can be concluded that State 1 and State 2 were successfully induced in the WT and *ΔLhca* plants, while *Stn7ΔLhca* plants were locked in State 1.

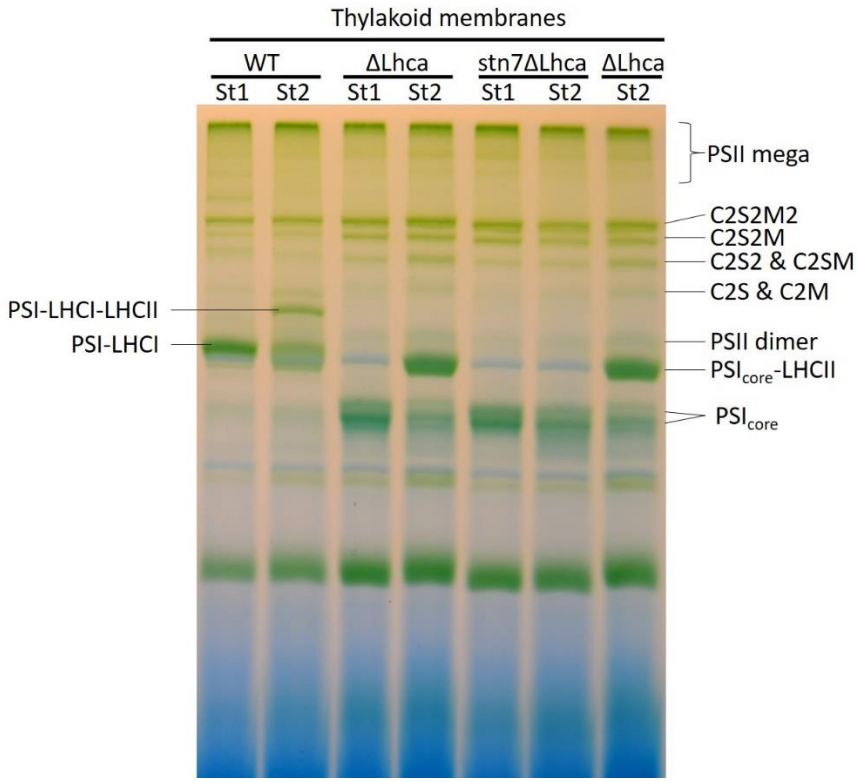

Figure 1. *Native PAGE of digitonin solubilized thylakoids.*

To evaluate the functional PSI antenna size in intact thylakoids, the P700 oxidation kinetics was followed by absorption spectroscopy (Figure 2 and table 1). In State 1 the PSI antenna size was 79 ± 5.4% for *ΔLhca* plants and 81± 6.6% for *Stn7ΔLhca* plants, relative to 100% for WT State 1 plants. The smaller PSI antenna size of *ΔLhca* plants is consistent with the lack of LHCI in this mutant. Moving to State 2 increased the PSI antenna size by 30 ± 9% in WT (table 1) and by 25 ± 8% in *ΔLhca.* The increase in antenna size of the *ΔLhca* plants is comparable to that of WT plants, showing that LHCII is still capable of enlarging the antenna size of PSI in the absence of LHCI. No change in the antenna size is observed for *Stn7ΔLhca* plants, as is expected in absence of Stn7.

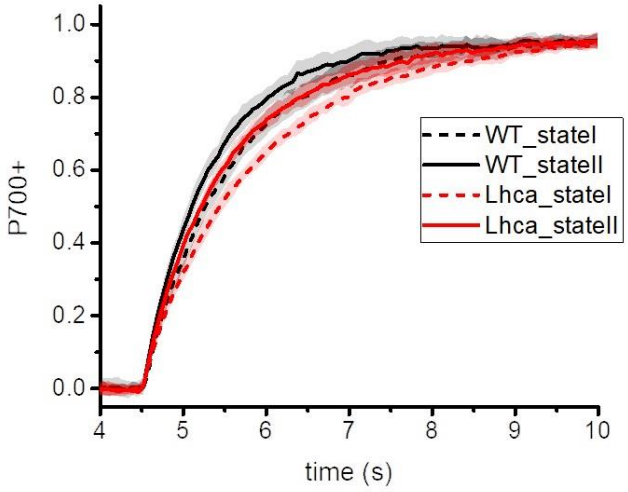

Figure 2. *Functional PSI antenna size in intact thylakoids. the P700 oxidation kinetics. P700 oxidation kinetics of WT and ΔLhca thylakoids in State 1 and State 2 was followed by absorption spectroscopy (λ=830-875 nm). The oxidation kinetics of Stn7ΔLhca thylakoids, in both far-red and orange adapted leaves (data not shown), overlapped with the ΔLhca State 1 thylakoids and is not shown here.*

SDS-PAGE analysis of stroma lamellae membranes – In the stroma lamellae almost all the light is harvested by PSI and its antenna, while PSII is only present at a very low level (Andersson and Anderson, 1980; Bressan et al., 2018). This membrane fraction is therefore suitable to investigate how many LHCII trimers are present per PSI and next to study the energy transfer from these LHCII complexes to PSI in the presence (WT) and absence (*ΔLhca*) of the LHCI system. Figure 3 shows the Lhc region upon SDS-PAGE fractionation of proteins from the State 1 and State 2 stroma lamellae. For WT and *ΔLhca* membranes the content of Lhcb1 and Lhcb2, which together form the LHCII trimer, is clearly increased in State 2 relative to State 1 membranes. In line with the greater abundance of chlorophyll *b*-rich LHCII complexes in State 2 membranes, the chlorophyll a/b ratio is decreased compared to State 1 (Figure 3). The SDS-PAGE analysis is used to quantify the number of LHCII trimers per PSI (Table 2).

Table 1. *PSI functional antenna size and chlorophyll a/b ratios of WT, ΔLhca and Stn7ΔLhca plants. For the chlorophyll a/b ratio the standard deviation of 3 measurements is indicated. The PSI antenna size is based on the P700 oxidation kinetics and expressed as % of the antenna size of WT State 1. The characters a-c indicate significantly different values (ANOVA, p<0.02), based on 5 repetitions per sample and 2 replica measurements.*

	WT	*ΔLhca*	*Stn7ΔLhca*
	Chlorophyll a/b ratio		
State 1	2.81 ± 0.11	3.07 ± 0.04	2.75 ± 0.09
State 2	2.84 ± 0.09	3.03 ± 0.08	2.78 ± 0.11
	PSI functional antenna size		
State 1	100 ± 5.6% [a]	79 ± 5.4% [c]	81 ± 6.6% [c]
State 2	130 ± 7.5% [b]	104 ± 5.8% [a]	81 ± 4.7% [c]

The PSI antenna system was already endowed with 0.7 LHCII trimers in WT and by 0.4-0.5 LHCII trimers in both *ΔLhca* and *Stn7ΔLhca* State 1 stroma lamellae membranes. Upon going to State 2, this ratio increased to 2.3 LHCII trimers/PSI in WT and 1.9 LHCII trimers/PSI in *ΔLhca* stroma lamellae. The increase of ~1.5 LHCII trimers in the membranes of both genotypes is far larger than the 0.5 that we observed before in *Arabidopsis* WT (Bos et al., 2019). In the present work detached leaves were fully exposed to the State 2 inducing light, while in our earlier work the whole plants were exposed. This likely induced a more extreme State 2 in the present work. In addition, differences in plant growing conditions might have further influenced the extent of LHCII relocation. Based on the native-PAGE gel we have concluded that ~0.5 LHCII trimers per PSI is associated at the PsaH/L/O site of the core in the digitonin stable PSI-LHCI-LHCII complex of WT State 2

thylakoids and almost 1 LHCII in the PSI_{core}-LHCII complex of *ΔLhca* thylakoids. Assuming that this ratio is similar in stroma lamellae it follows that roughly 1.8 digitonin-sensitive LHCII per PSI is present in WT and 0.9 LHCII per PSI in *ΔLhca* State 2 stroma lamellae. Surprisingly, a small increase of 0.3 LHCII trimers per PSI was also observed for *Stn7ΔLhca* stroma lamellae. As no PSI_{core}-LHCII complex is formed in this mutant (Figure 1), this difference is entirely due to digitonin-sensitive LHCII complexes.

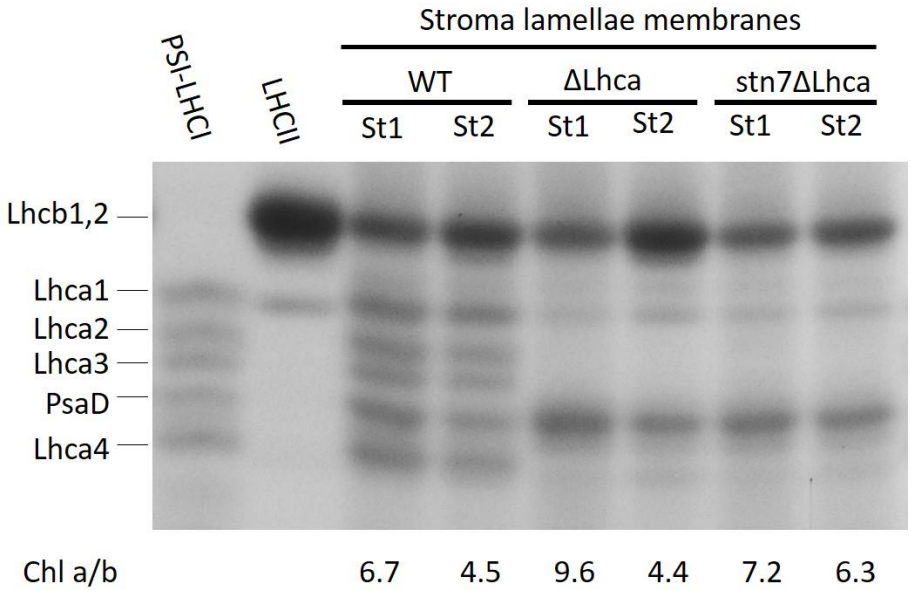

Figure 3. *SDS-PAGE gel and chlorophyll a/b ratio of WT, ΔLhca and Stn7ΔLhca stroma lamellae membranes. The region of the Lhcs and the PsaD PSI core polypeptides is shown, as indicated in the figure. Contrast is enhanced for improved clarity.*

Excitation-energy transfer and trapping in stroma lamellae membranes – Streak-camera measurements allow to follow the fluorescence intensity with picosecond time-resolution as a function of emission wavelength. A fast decay of the fluorescence from PSI generally means that the excitation-energy is quickly transferred to the reaction center (RC), where it is trapped (Russo et al., 2020). The faster the energy is trapped by the RC the lower the possibility that the energy is lost by other competing processes and as such the higher the quantum yield of the photosystem.

Table 2. *Number of LHCII trimers per PSI in WT, ΔLhca and Stn7ΔLhca stroma lamellae membranes based on the SDS-PAGE gel. The standard deviation of two technical replicates is presented.*

LHCII trimer/PSI	WT	*ΔLhca*	*Stn7ΔLhca*
State 1	0.7 ± 0.0	0.4 ± 0.1	0.5 ± 0.0
State 2	2.3 ± 0.1	1.9 ± 0.3	0.8 ± 0.0
State 2 – State 1	1.5 ± 0.1	1.5 ± 0.3	0.3 ± 0.0

The fluorescence of PSI core complexes from plants decays in 20-25 ps (Slavov et al., 2008; Wientjes et al., 2011), which is two orders of magnitude faster than the ns decay of the isolated Lhc antenna, indicating that the quantum yield of charge separation is close to unity. The fluorescence lifetime of PSI-LHCI is longer due to three reasons. First, the number of chlorophyll *a* molecules per RC has increased, which leads to a longer average migration time from the antenna chlorophylls to the RC (van Grondelle and Gobets, 2004; Broess et al., 2006). Second, the probability that the excitation is located on the RC is lower for a larger antenna system, thus increasing the trapping time (Broess et al., 2006). Third, Lhca3 and Lhca4, which are part of LHCI, contain so called red-forms, chlorophylls which absorb light at longer wavelengths (lower energy) than the RC. The slow up-hill energy transfer from the red-forms to the RC further increases the fluorescence lifetime (Jennings et al., 2003). Nevertheless, the average lifetime of plant PSI-LHCI is only 50-60 ps (Croce et al., 2000; Ihalainen et al., 2005; Slavov et al., 2008; van Oort et al., 2008; Wientjes et al., 2011; Croce and van Amerongen, 2020), showing that its efficiency is still over 98%.

In order to investigate the role of LHCI in mediating energy transfer from LHCII to PSI, we performed streak-camera fluorescence decay measurements on WT and *ΔLhca* stroma lamellae membranes. In figure 4A the fluorescence intensity is depicted by the color scale, the vertical axis shows the time and the horizontal

axis the wavelengths. Along the horizontal axis, one can see how the fluorescence spectrum at different time points after the excitation; as an example the white dashed line shows the average fluorescence spectrum 70-90 ps after the laser pulse (indicated by the white dashed box). This spectrum of WT State 1 stroma lamellae membranes shows a strong shoulder around 715 nm, typical for PSI-LHCI. In WT State 2 membranes the intensity of the peak at 680 nm is increased, in agreement with the increased LHCII content. In the *ΔLhca* membranes the shoulder around 715 nm is absent due to the lack of the red forms of Lhca3 and Lhca4 (Croce et al., 2002). Along the vertical axis the fluorescence intensity is followed over time. As an example figures 4B,C show the fluorescence decay traces of the WT and *ΔLhca* membranes around 682 nm and 715 nm. In WT stroma lamellae membranes the fluorescence decay around 715 nm is slower than around 682 nm, which can be readily explained by the slow up-hill energy transfer from the red forms (around 715 nm) to the bulk chlorophylls (around 682 nm) (Jennings et al., 2003; Wientjes et al., 2011; Wientjes et al., 2011). Instead the decay kinetics are almost independent on the wavelengths for the *ΔLhca* membranes. Increasing the PSI antenna size with LHCII in State 2 slows down the fluorescence decay kinetics in both WT and *ΔLhca* membranes, showing that it takes on average more time to transfer the excitation energy to the RC.

To make a quantitative comparison of the excitation-energy transfer and trapping kinetics the data is described with decay-associated spectra (DAS). For each wavelength the fluorescence decay is described with a sum of exponentials: $F(t) = a_1 e^{-t/\tau 1} + a_2 e^{-t/\tau 2} + \cdots$, in which the lifetimes (τ_n) are the same for each wavelength and the amplitudes (a_n) are plotted as the DAS, showing how much each lifetime contributes to the fluorescence decay at that wavelength. In figure 5 the DAS of WT and *ΔLhca* stroma lamellae after 400 nm nm excitation are compared to the decay of isolated PSI-LHCI (from (Bos et al., 2017)) and PSI core complexes (see Materials and Methods). All samples have a spectrum associated with a short ~5 ps lifetime, with both a maximum around 680-690 nm and a minimum around ~715 nm. This represents excitation-energy equilibration between bulk chlorophylls *a* and the low-energy emitting chlorophylls *a* (red-forms) of the

PSI core and especially of Lhca3 and Lhca4. In PSI-LHCI the fluorescence decays with two lifetimes, a 26 ps component with a maximum at 680-690 nm and an 86 ps component with a red-shifted emission maximum of ~ 715 nm. The WT State 1 stroma lamellae with 0.7 LHCII trimers per PSI show increased emission around 680 nm in the 37 ps and 121 ps DAS when compared with the 26 ps and 86 ps DAS of isolated PSI-LHCI complexes. Indeed, an increase in amplitude at 680 nm is expected as this is the emission maximum of LHCII. Furthermore, all stroma lamellae DAS show a ~1 ns component with a maximum at 680 nm, which is ascribed to PSII core complexes which are known to be present in stroma lamellae membranes (Andersson and Anderson, 1980).

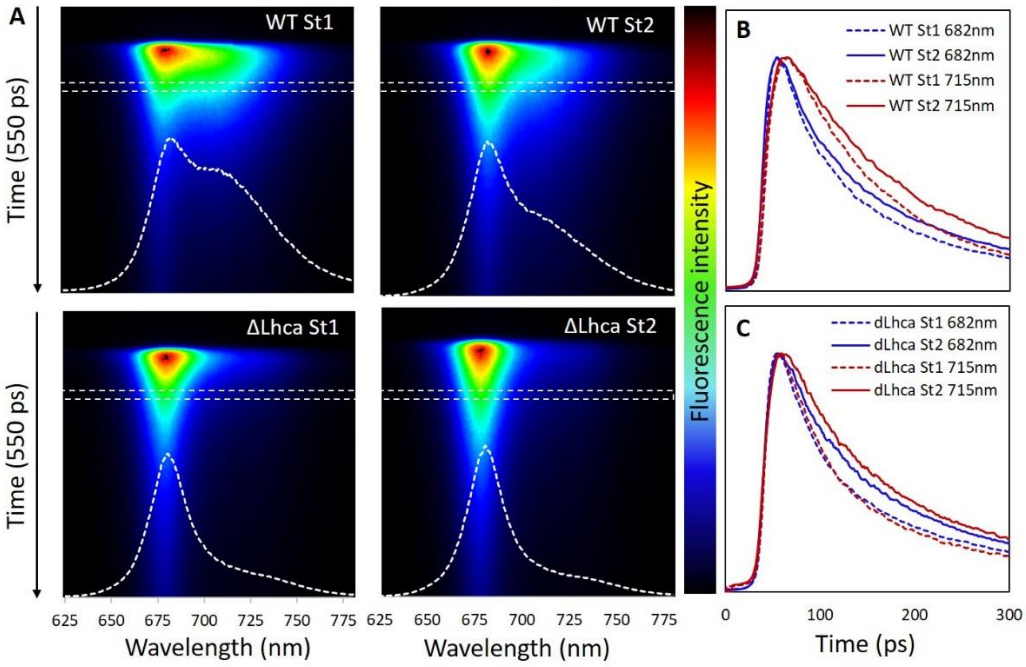

Figure 4. *Streak-camera fluorescence decay measurements of WT and ΔLhca State 1 and State 2 stroma lamellae membranes. Excitation was at 400nm. A. Streak-camera images and the fluorescence spectra 70-90 ps after the laser pulse. B,C. Fluorescence decay kinetics of WT (B) and ΔLhca (C) State 1 and State 2 membranes at 682±3 nm and 715±3 nm. The fluorescence decay of State 2 membranes is slower than that of State 1 membranes for both WT and ΔLhca stroma lamellae.*

Upon transitioning to State 2 the number of LHCII complexes per PSI increase to 2.3, this results in a further relative increase in the DAS around 680 nm. The average PSI-LHCII$_n$ fluorescence lifetime increases from 89±8 ps in WT State 1 stroma lamellae membranes to 102±4 ps in State 2 (Table 3). The average increase in lifetime (State 2 – State 1) is 14±4 ps (Table 3), which can be ascribed to the increase in antenna size with 1.5 LHCII trimers (Table 2). This increase of the lifetime is due to the increased trapping time plus the LHCII to PSI migration time (Broess et al., 2006). Based on the absorption spectra and the number of chlorophylls per complex the additional LHCIIs absorb 25% of the light at 400 nm (Figure 6). If all excitation were created on LHCII the increase in lifetime would have been $\frac{14\pm4}{0.25} = 55 \pm 16\, ps$ (van Oort et al., 2008). This sets the limit for the average time it takes to transfer excitation-energy from the extra LHCIIs to PSI to a maximum of 55 ± 16 ps.

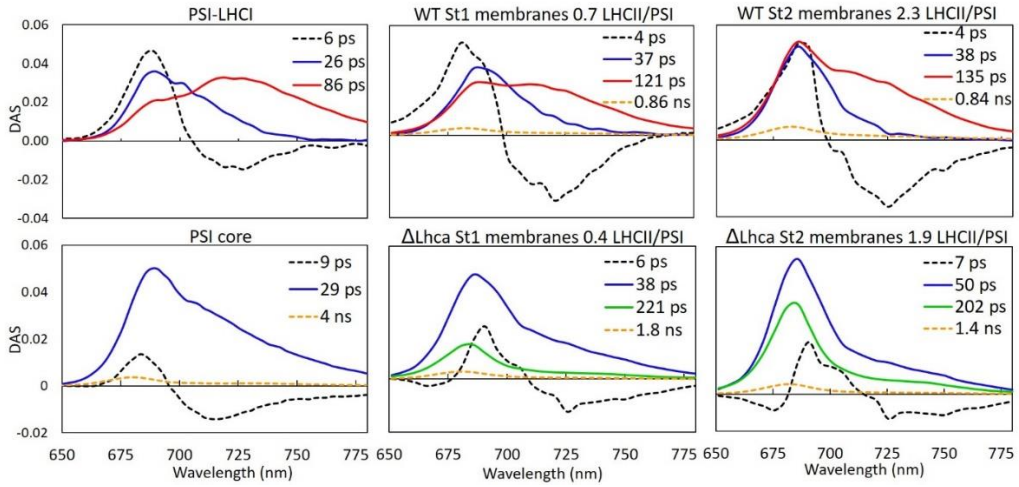

Figure 5. *Decay associated spectra (DAS) of PSI-LHCI, PSI core, State 1 and state 2 WT and ΔLhca stroma lamellae membranes. Excitation was at 400 nm. The number of LHCII complexes per PSI, based on the SDS-PAGE gel, is indicated in the figure.*

Table 3 Average fluorescence lifetime of PSI-LHCII complexes in the stroma lamellae. *The average fluorescence lifetimes were calculated based on the relative area of the DASs colored red and blue in figure 5, whereas the ~1 ns lifetime attributed to PSII core fluorescence was not taken into account. The standard deviation is provided based on 3 measurements; the plants were grown twice and stroma lamellae membranes were isolated. One set of samples was measured twice on different days and the other set was measured ones. The difference in lifetimes between State 1 and State 2 was calculated based on one set of measurements and then averaged for the three replicates. Excitation was at 400 nm.*

Average lifetime	WT	ΔLhca	Stn7*ΔLhca
State 1	89±8 ps	67±10 ps	75±9 ps
State 2	102±4 ps	93±14 ps	90 ± 10 ps
State 1 – State 2	14±4 ps	34±4 ps	15±3 ps

The fluorescence decay of the PSI core could be described with one lifetime of 29 ps. A small 4 ns contribution was also present, but this is attributed to a contamination of uncoupled light-harvesting complexes and/or free chlorophylls. In the *ΔLhca* State 1 stroma lamellae membranes the PSI core antenna size is enlarged by 0.4 LHCII trimers. This LHCII contributes mainly to the new DAS with a lifetime of 221 ps. In State 2 the shortest trapping lifetime increases from 29 ps to 50 ps, clearly indicating that LHCII contributes to this component. In addition, a 200 ps DAS strongly contributes to the decay (Figure 5). As shown in WT, in State 2 *ΔLhca* membranes the LHCII/PSI ratio is increased by 1.5 LHCII trimers compared to State 1. LHCII is responsible for 33% of the 400 nm light absorption in the PSI_{core}-$LHCII_{1.9}$ complex. The average lifetime of State 2 compared to State 1 membranes is 34±4 ps longer (Table 3). It can thus be estimated that the sum of the increased trapping time and the transfer time from the additional LHCIIs to the

PSI core is $\frac{34\pm4}{0.33} = 104 \pm 16\, ps$. The DAS of State 1 and State 2 *Stn7ΔLhca* membranes look very similar to that of *ΔLhca* State 1 membranes, with a small increase in the amplitude of the ~ 200 ps component in State 2 (data not shown).

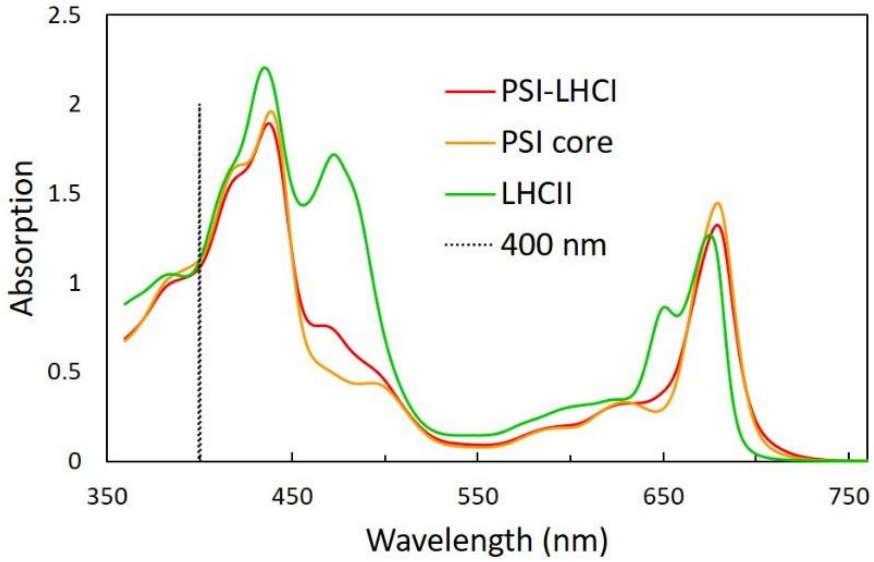

Figure 6. Absorption spectra of PSI-LHCI, PSI core and LHCII. *Spectra are normalized to the same number of chlorophylls as described in Materials and Methods.*

Discussion

How fast and efficient does LHCII transfer energy to PSI? – In recent years evidence accumulated which suggests that LHCII operates as PSI antenna, not only in State 2, but also in State 1 (Benson et al., 2015; Grieco et al., 2015; Bressan et al., 2018; Bos et al., 2019; Chukhutsina et al., 2020). In this work we investigated how efficient LHCII transfers energy to PSI in stroma lamellae membranes, with the emphasis on the role of LHCI in mediating energy transfer from LHCII to PSI. In the *ΔLhca* membranes, devoid of LHCI, a decay component with a lifetime of 221 ps was attributed to LHCII. The total decay rate of 1000/221 = 4.5 ns^{-1} is the sum of the LHCII to PSI transfer rate and the intrinsic LHCII decay rate ($k_{tot} = k_{transfer} + k_{intrin\,decay}$). The intrinsic decay rate of LHCII in a membrane is hard to determine as it strongly depends on its aggregation state. Lifetimes between 1 ns and 3 ns were measured for LHCII in liposomes (corresponding to rates of 1 to 0.33

ns^{-1}), depending on the protein to lipid ratio (Natali et al., 2016), and an average rate of 1.7 ns^{-1} has been determined for disconnected LHCIIs in the CP24 knock-out mutant (van Oort et al., 2010) and for lamellar LHCII aggregates (Miloslavina et al., 2008). As we assume that the LHCII complexes in the stroma lamellae are not aggregated we will use a value of 0.5 ns^{-1}, as it has been used before in LHCII to PSI energy transfer studies (Akhtar et al., 2016; Santabarbara et al., 2017). This gives a rate of: $k_{transfer} = k_{tot} - k_{intrin\ decay} = 4.5 - 0.5 = 4ns^{-1}$ (or a transfer time of 250 ps), for the unphosphorylated LHCII to PSI core energy transfer in State 1 *ΔLhca* stroma lamellae. In State 2 *ΔLhc*a membranes, the PSI antenna size is increased by 1.5 LHCII trimers to 1.9 LHCII/PSI core, of which one is present in the digitonin stable PSI_{core}-LHCII complex. The other 0.9 LHCII likely interact with the PSI core on other, yet unidentified, location(s). The newly arising 50 ps decay component (Figure 5) is reminiscent of the main 58 ps decay component observed for digitonin isolated PSI_{core}-LHCII complexes after excitation with 475 nm light, which is strongly absorbed by LHCII (Bressan et al., 2016). It can therefore be assigned to the energy transfer from the phosphorylated $Lhcb1_2Lhcb2$ trimer to the PsaH/L/O site of the PSI core and occurs with a rate of: $k_{transfer} = k_{tot} - k_{intrin\ decay} = \frac{1000}{58} - 0.5 = 16.7\ ns^{-1}$ (transfer time of 60 ps). This is consistent with previous energy transfer studies on isolated PSI-LHCI-LHCII complexes which showed an increased amplitude in the 30-40 ps and 80-100 ps components, relative to PSI-LHCI (Galka et al., 2012; Wientjes et al., 2013). Similar transfer times in the order of 60 ps where observed for LHCII to PSI transfer in spinach PSI-LHCII membranes (Bos et al., 2017) and in PSI-LHCI-$Lhcb_7$ supercomplexes from *Chlamydomonas* (Le Quiniou et al., 2015). It is also in agreement with the 15 ns^{-1} transfer rate observed for 20% of the LHCII to PSI transfer based on kinetic modelling of isolated PSI-LHCI-LHCII decay (Santabarbara et al., 2017). However, it is considerably slower than the main LHCII to PSI transfer component of 55 ns^{-1} found in the same study (Santabarbara et al., 2017). The other, "additional" LHCII trimer present in the State 2 *ΔLhca* membranes contributes to the ~200 ps decay component, which corresponds to a transfer rate of 4.5 ns^{-1} (transfer time of 222 ps). This is similar to the transfer rate

of 3 ns^{-1} observed in reconstituted LHCII:PSI liposomes (Akhtar et al., 2016). The average transfer rate of the 1.5 extra State 2 LHCIIs is 2/3*16.7+1/3*4.5=12.6 ns^{-1}, in good agreement with an average transfer rate of at least 9.6 ns^{-1} (inverse of 104 ps lifetime) which was determined in the results section based on the increased absorption cross section and average lifetimes.

For WT stroma lamellae it is not possible to extract the LHCII to PSI-LHCI transfer times directly from the DAS as they are very similar to the PSI-LHCI trapping times and are therefore not resolved separately. However, based on the increase in the average lifetime upon State 1 to State 2 transition, it was calculated that the sum of the increased trapping time and the LHCII to PSI migration time was 55±16 ps, meaning that the 1.5 extra LHCII complexes transfer their energy to PSI in less than 55±16 ps. In State 2 ~0.5 LHCII trimers are associated on the PsaH/L/O site of the PSI core and ~1 LHCII trimer at another location. As the PsaH/L/O binding trimer transfers with a lifetime of 60 ps (*vide supra*) this means that the other LHCII trimer should transfer with a lifetime of less than 55 ps. The transfer from the "additional" LHCIIs to the PSI core in absence of LHCI (ΔLhca mutant) took at least 4 times longer. This strongly suggests that LHCI has a role in mediating energy transfer from LHCII to PSI, most likely by having specific LHCII binding sites which allow for short Chlorophyll – Chlorophyll distances, favorable for Förster resonance energy transfer. Instead the energy transfer from "additional" LHCII complexes to PSI in the *ΔLhca* mutant is probably caused by random interaction with the core, similar to what happens when PSI and PSII mix and excitation energy spills over from PSII to PSI (Anderson, 1999). Figure 7 gives a schematic overview of the transfer rates and number of LHCII trimers per PSI in WT and *ΔLhca* stroma lamellae of WT and *ΔLhca* plants. In WT plants the "additional", non PsaH/L/O binding, LHCII trimers are suggested to interact with LHCI. Likely locations are Lhca2, as has been observed with electron microscopy (Yadav et al., 2017) and possibly on Lhca1. These Lhca's have in common that they do not locate chlorophylls with absorption maxima above 700 nm (Croce et al., 2002). Due to the absence of the low-energy chlorophyll, the speed of energy transfer from Lhca1 and Lhca2 to the PSI-core is expected to be much faster, than that of Lhca3 and Lhca4 (Wientjes et al., 2011). As such the excitation energy

harvested by LHCII can be transferred more efficiently to the reaction center when associated with Lhca1 and Lhca2.

The efficiency of the LHCII to PSI excitation-energy transfer can be calculated based on the transfer rate and the total decay rate: $Transfer\ efficiency = k_{transfer}/k_{tot}$. This gives a transfer efficiency of 97% for the PsaH/L/O binding LHCII trimer and of 90% for the "additional" LHCII trimers in the *ΔLhca* mutant.

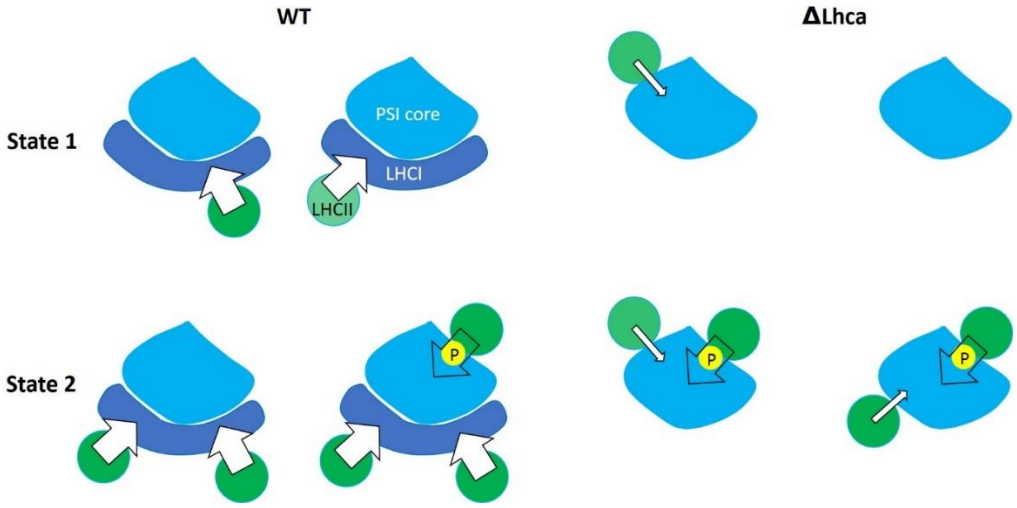

Figure 7. *Rate of energy transfer from LHCII to PSI in State 1 and State 2 stroma lamellae membranes from WT and ΔLhca plants. The width of the arrow represents the rate of the energy transfer. The number of LHCII trimers per PSI complex is based on the SDS-PAGE data. Color coding: green – LHCII trimer, light green – LHCII trimer present in a fraction of the complexes, P in yellow circle – phosphate group on Lhcb2 associating with the PSI core, light blue – PSI core, dark blue – LHCI.*

How much are state transitions affected in the ΔLhca mutant? – When evaluating state transitions with a Pulse-Amplitude-Modulation (PAM) chlorophyll fluorometer, the change in the PSII antenna size by state transitions is reduced to 31% (Benson et al., 2015) or even 13% (Bressan et al., 2018) in the *ΔLhca* mutant relative to WT levels. Instead, judged by the P700 oxidation kinetics the change in the PSI antenna size in the *ΔLhca* mutant is only reduced to 61% (Benson et al., 2015) or 83% (this work). Even more, the increase in number of LHCII complexes

per PSI upon transitioning from State 1 to State 2 is the same for the stroma lamellae membranes from *ΔLhca* and WT plants. These seemingly contradictory observations probably arise from a combination of at least two factors. First, the far-red 720 nm LED source used in the PAM to over-excite PSI is probably not strong enough to fully induce State 1 in *ΔLhca* plants, as they lack most of the red-forms which are located in LHCI. This light source will be especially ineffective when used in combination with orange or red light, which over-excites PSII as used by (Benson et al., 2015; Bressan et al., 2018). Instead, the change in the PSI antenna size was estimated from measurements on thylakoids that were isolated from leaves illuminated by far-red light only for at least 45 min, to induce State 1, or red/orange light to induce State 2 (ref (Benson et al., 2015) and this work). Second, the relocation of LHCII during state transitions could be affected in the *ΔLhca* mutant by a change of the thylakoid ultrastructure (Bressan et al., 2018). A previous membrane fractionation study showed that in WT plants the transition from State 1 to State 2 resulted in an increase of LHCII in the margins and stroma lamellae fraction at the cost of LHCII in the grana fraction. Instead, in *ΔLhca* plants LHCII only moved from the grana to the stroma lamellae fraction (Bressan et al., 2018). Taken together, it can be concluded that the presence of LHCI is not a requirement for the successful relocation of LHCII between grana and stroma lamellae membranes, nor for excitation-energy transfer from mobile LHCII to the PSI core.

Regulation of LHCII movement – This work further supports the recent observations that in State 1 unphosporylated LHCII enlarges the antenna of PSI in *A. thaliana* (Benson et al., 2015; Bressan et al., 2018; Bos et al., 2019). Future research needs to point out how the distribution of this unphosphorylated LHCII between the grana and stroma lamellae membranes is regulated. One possible mechanism is the reversible acetylation of LHCII lysines. Acetylation of a lysine neutralizes the positive charge as such increasing the net negative charge of the LHCII surface. The increased negative charge of phosphorylated LHCII is one of the suggested mechanisms which drives the dissociation of LHCII from PSII, after which it can move to PSI (Allen, 1992). It has already been shown that lysine acetylation plays a role in state transitions, as *A. thaliana* plants which lack the lysine

acetyltransferase, NSI, cannot form the PSI-LHCI-LHCII supercomplex under state 2 conditions (Koskela et al., 2018).

In conclusion: LHCI is not required for the "additional" non PsaH/L/O binding LHCII trimers to transfer energy to PSI; however LHCI does have a role as docking site for these "additional" LHCIIs and allow for efficient excitation-energy transfer to the PSI core.

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Conclusion

In this thesis I've shown that heterologous expression of LHCSR1 in *A. thaliana npq4* mutant yields a pigment-binding protein with properties reproducing those of LHCSR1 from the homologous system *P. patens*. The protein is active in NPQ, yet the induction requires sustained light treatment due to the need for Zea build-up. Reasons for a decreased NPQ include (a) insufficient Zea accumulation in *A. thaliana* with respect to *P. patens* for full NPQ activity and (b) the localization of the protein in the stromal membranes of thylakoids which is rich in highly fluorescent LHCII in mosses but not in plants (Pinnola et al. 2015a). The level of quenching in *npq2npq4*+LHCSR1 (endowed with full Lut and Zea levels) recovered up to 70% with respect to *A. thaliana* WT, proving that LHCSR1 can be highly functional in vascular plants. Furthermore, I prove that this system is sensitive to physiological differences which makes *A. thaliana* an excellent organism for the analysis of LHCSR activity. Indeed, I could assess that Lut was not an absolute requirement for *in vivo* quenching in LHCSR1, since quenching activity was obtained in *lut2npq4*+LHCSR1 plants.

I then used this system to analyse possible interaction partners of LHCSR1, by complementing mutants lacking specific antenna complexes. I conclude that neither Lhcb4 nor Lhcb6 is involved in the quenching activity of LHCSR1. However, due to the low expression levels of LHCSR1 in the absence of *lhcb5* I was unable to determine whether the interaction partner is Lhcb5 and/or LHCII as previously suggested (Pinnola et al. 2015a; Semchonok et al. 2017). Interestingly there seems to be a connection between the absence of the *lhcb5* gene and the expression level of *lhcsr1* in the moss *P. patens* similar to what I observed in the *A. thaliana* mutants (Peng et al. 2019). Moreover, it seems that the NPQ-kinetics of the *lhcb5* knockout mutant in *P. patens* are almost identical to the *lhcsr1* knockout (Alboresi et al. 2010; Peng et al. 2019), thereby suggesting that *lhcb5* is essential for both the accumulation and the interaction with LHCSR1.

In order to improve the yield of crops it will be necessary to fine-tune the photoprotective mechanisms to ensure minimal energy dissipation while maintaining enough photoprotection. This will lead to different levels and probably

different types of photoprotection, depending on the growth conditions. Plants in greenhouses are relatively well protected against high light, drought and cold, while plants in the field might need to be more drought resistant and cope with fluctuating high light. Therefore I proceeded to test whether the *npq4*+LHCSR1 lines could replace PSBS or could increase the biomass production in specific conditions. However, LHCSR1 was not able to completely replace PSBS, although in certain conditions there was a clear advantage to plants expressing LHCSR1 in comparison to the *npq4* mutant. LHCSR1 seemed important in conditions where an additional abiotic stress (cold) was added in addition to the fluctuating high light, but it is not yet clear whether the small contribution of NPQ was more important than a real protection against lower temperatures. Finally, since the NPQ activity of LHCSR1 and PSBS is cumulative, plants expressing both PSBS and LHCSR1 might prove to be more resilient in extreme conditions, future work will need to elucidate if this also can lead to increased biomass production.

Another important factor in a good biomass production is a well-balanced excitation pressure on both photosystems. This is regulated by the shuttling of LHCII between PSII and PSI, it is known that phosphorylated LHCII binds to PSI at the PsaL/O/H site. However, recent observations indicate that in State 1 unphosporylated LHCII enlarges the antenna of PSI in *A. thaliana* (Benson et al., 2015; Bressan et al., 2018; Bos et al., 2019). In the last chapter of this work I show that LHCI is important for the efficient energy transfer of unphosphorylated LHCII to the PSI-core.