Function and regulation of PsbS in *C. reinhardtii*

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Fritz Hemker

from Münster, Germany

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from the Institute for Plant Biochemistry
at the Heinrich Heine University Düsseldorf

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Supervisor: apl. Prof. Dr. Peter Jahns
Co-supervisor: Prof. Dr. Georg Groth

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STATEMENT OF DECLARATION

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1. SUMMARY

Chlamydomonas reinhardtii is a unicellular green alga that has the potential to serve as a multifaceted modern-day crop. For efficient agriculture of *C. reinhardtii*, it is crucial to understand in detail photosynthesis and stress defense including the underlying molecular processes. Under field conditions, photosynthetic organisms are frequently exposed to rapidly varying and often harmful light intensities, so that efficient photoprotection is essentially required for survival. Efficient photoprotection is ensured by a network of different physiological and molecular mechanisms. One of the most powerful mechanisms is non-photochemical quenching (NPQ) of excitation energy that involves multiple strategies to dissipate excess light energy as heat while still enabling growth via photosynthesis. In *C. reinhardtii*, the dominating quenching process is termed qE, the energy-dependent component of NPQ. qE is based on the pH-regulated activation of LHCSR proteins, which in turn induce quenching of excitation energy through interaction with LHCII proteins of PSII. Vascular plants have lost the genetic information on LHCSR proteins, but the PsbS protein adopted the function as pH-regulator of qE. Interestingly, *C. reinhardtii* possesses an ortholog of PsbS that is similarly regulated as the LHCSR proteins. However, the precise function of PsbS in *C. reinhardtii* remains unknown. PsbS accumulates within a few hours upon acclimation to high light (HL) intensities but is degraded after 8-16 h of HL exposure. These characteristics led to the assumption that PsbS might be required for qE activation in the early stages of HL acclimation. In this thesis, the regulation pattern and the possible function of PsbS in *C. reinhardtii* in photoprotection were analyzed under various conditions. In fluctuating light conditions, PsbS degradation was found to be reduced compared to continuous light, indicating that rapid changes of the light intensity influence PsbS accumulation. Moreover, the stress level of *C. reinhardtii* was enhanced and both the pool size and the de-epoxidation state of the xanthophyll cycle pigments were altered under those conditions. Combining high light and salt stress increased PsbS accumulation in comparison with control conditions. Again, this enhanced accumulation was accompanied by higher stress levels and an increased de-epoxidation state of the xanthophyll cycle pigments. Finally, analysis of PsbS knock-out mutants clarified that PsbS has no immediate influence on qE or fitness under constant or fluctuating light conditions but might rather impact the structure and fluidity of thylakoid membranes, as derived from altered xanthophyll conversion. This conclusion agrees with an enhanced accumulation of PsbS under salt stress conditions where the remodeling of the thylakoid membrane is a crucial defense mechanism. Even though PsbS has clearly different functions in qE and different accumulation patterns in vascular plants and green algae, the regulation of thylakoid membrane organization is likely a central property of PsbS which remained unchanged during evolution.

2. ZUSAMMENFASSUNG

Chlamydomonas reinhardtii ist eine einzellige Grünalge mit dem Potential, eine vielseitige Produktionsplattform zu werden. Um *C. reinhardtii* effizient anzuziehen, ist es wichtig, Photosynthese und Stressreaktionen im Detail zu verstehen, einschließlich der zugrunde liegenden molekularen Prozesse. Unter Freilandbedingungen sind photosynthetische Organismen regelmäßig schnell variierenden und häufig schädlichen Lichtintensitäten ausgesetzt. Daher ist effiziente Photoprotektion entscheidend für deren Überleben. Diese effiziente Photoprotektion wird durch ein Netzwerk verschiedener physiologischer und molekularer Mechanismen sichergestellt. Eine der effektivsten Methoden ist dabei die nicht-photochemische Löschung (NPQ) von Anregungsenergie, welche mehrere Mechanismen und Strategien umfasst, die überschüssige Lichtenergie in Form von Wärme abzugeben, ohne dabei die Nutzung der Photosynthese für das Wachstum zu beeinträchtigen. In *C. reinhardtii* ist der dabei dominierende Prozess der sogenannte qE-Mechanismus, die energieabhängige Komponente des NPQ. Der qE-Mechanismus beruht auf der pH-regulierten Aktivierung der LHCSR Proteine, welche die Löschung überschüssiger Anregungsenergie durch Interaktion mit LHCII Proteinen induzieren. Gefäßpflanzen verwenden statt LHCSR Proteinen das PsbS Protein für den gleichen Mechanismus. Interessanterweise besitzt *C. reinhardtii* ebenfalls ein Ortholog des PsbS, das ähnlich reguliert wird wie LHCSR. Bis heute ist die genaue Funktion von PsbS in *C. reinhardtii* allerdings noch nicht aufgeklärt. PsbS akkumuliert innerhalb weniger Stunden bei der Anpassung an Starklichtbedingungen und wird aktiv nach ungefähr 8 bis 10 Stunden im Starklicht abgebaut. Diese Dynamik hat zu der Annahme geführt, dass PsbS in den frühen Phasen der qE Aktivierung eine Rolle spielen könnte. In dieser Arbeit wurde die Regulation und potenzielle Funktion von PsbS unter unterschiedlichen experimentellen Bedingungen untersucht. Dabei zeigte sich, dass der Abbau von PsbS unter fluktuierenden Lichtbedingungen im Vergleich mit kontinuierlicher Belichtung vermindert wurde, vor allem bei schnellen Wechseln von Dunkelheit und Starklicht. *C. reinhardtii* zeigte unter diesen Bedingungen einen erhöhten Stresszustand und sowohl die Menge an Xanthophyllzykluspigmenten als auch deren De-epoxidationszustand waren erhöht. Zudem wurde eine stark erhöhte Akkumulation von PsbS bei kombiniertem Starklicht- und Salzstress festgestellt. Dabei wurde erneut eine Erhöhung des Stresslevels und des De-epoxidationsstatus der Xanthophyllzykluspigmente festgestellt. Des Weiteren zeigten zwei PsbS Knock-out Mutanten, dass PsbS keinen direkten Einfluss auf qE oder die Fitness der Zellen unter den verwendeten konstanten oder fluktuierenden Bedingungen hat. Stattdessen wies die veränderte Umwandlung der Xanthophylle darauf hin, dass PsbS wahrscheinlich die Membranstruktur und/oder -fluidität verändert. Diese Schlussfolgerung wird von dem Befund

unterstützt, dass der PsbS Gehalt unter Salzstress erhöht war, wodurch der Umbau der Thylakoidmembran gefördert werden könnte, der unter Salzstress eine wichtige Akklimatisierungsstrategie darstellt. PsbS in Grünalgen zeigt im Vergleich zu höheren Pflanzen also deutliche Unterschiede in Bezug auf Regulation und Funktion des qE, aber die Regulation der Organisation der Thylakoidmembran scheint eine zentrale, gemeinsame Eigenschaft zu sein.

3. INTRODUCTION

Due to climate change and increasing world population, ensuring global nutrition becomes increasingly difficult. The agricultural sector faces trouble as crop yields are oftentimes very much affected by extreme weather events and increasing temperatures, coupled with other potential abiotic stressors. Nowadays, farmers and scientists have to find solutions for periods of excessive sunlight, storms, soil salinity, drought and much more aside from extreme temperatures. All those challenges increase the desire for promising crop alternatives. An organism group that shows immense potential as a production platform for valuable products and evades a lot of the challenges for established crops are unicellular green algae like *Chlamydomonas reinhardtii*.

Chlamydomonas reinhardtii

Unicellular green algae are exploited as food additives and superfoods (Becker, 2007; Pulz & Gross, 2004), as production platform for highly valuable proteins (Spolaore et al., 2006) or molecular hydrogen and biofuels (Chisti, 2007). In contrast to most plants, *C. reinhardtii* is able to grow heterotrophically, under laboratory conditions mostly on acetate as carbon source. This is a huge advantage in biotechnological application of *C. reinhardtii* as the doubling time increases significantly in heterotrophic conditions and yields become independent of environmental conditions. Aside from biotechnological application, *C. reinhardtii* has been established for decades as a model organism for the analysis of fundamental cellular processes such as photosynthesis. A model of the cell structure of *C. reinhardtii* is shown in Fig.1. *C. reinhardtii* cells are about 10 µm in length and contain only one large chloroplast in which thylakoids stack as lamella. An eyespot enables light detection, and two cilia offer motility to cells. In addition, *C. reinhardtii* possesses a nucleus and multiple mitochondria as well as a vacuole. In comparison to plant cells, the presence of the eyespot and cilia and the smaller vacuole in *C. reinhardtii* are among the most striking differences.

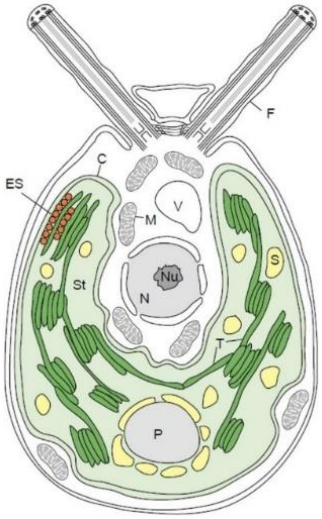

Figure 1: Model of *C. reinhardtii*. Chloroplast (C), eye spot (ES) flagella (F), mitochondria (M), nucleus (N), nucleolus (Nu), pyrenoid (P), starch grains (S), stroma (St), thylakoid membranes (T), vacuole (V). From (Jokel, 2017)

C. reinhardtii belongs to the green algae and specifically to the clade of Chlorophyta. The divergence between the Chlorophyta and the Streptophyta, which include all land plants, is estimated to have taken place more than one billion years ago (Leliaert et al., 2011). The process of photosynthesis has remained largely unchanged in plants and algae despite the excessive timespan of independent evolution of the two clades. Still, there are some crucial differences related to photosynthetic light utilization that are described in the following, with a special focus on the structure of photosystems, light harvesting and light energy dissipation that have been subject to changes in evolution (Table 1).

Table 1: Key differences between vascular plants and *C. reinhardtii*. The attributes were confined to relevant elements discussed in the present thesis.

Attribute		Vascular plants	*C. reinhardtii*	Sources
Cell structure		Large vacuole, multiple chloroplasts	Cilia, eyespot, single chloroplast	
Thylakoid membrane		Grana and lamellae	Appressed and non-appressed regions	(Wietrzynski et al., 2020)
PSII-LHCII	**Additional PSII subunits**		Ycf12, PsbR	(Sheng et al., 2019)
	Minor LHCIIs	CP24, CP26, CP29	CP26, CP29	(Elrad & Grossman, 2004)
	Major LHCIIs	$C_2S_2M_2$ (*Pisum sativum*)	$C_2S_2M_2L_2$	(Sheng et al., 2019; Su et al., 2017)
PSI-LHCI	**LHCI**	One ring of four LHCI proteins	Two rings of four LHCI proteins each; two additional LHCIs at the other side of the complex	(Ben-Shem et al., 2003; Ozawa et al., 2018)
NPQ	**qE regulator**	PsbS by sensing luminal acidification	LHCSR3 and LHCSR1 by sensing luminal acidification	(Fan et al., 2015; Peers et al., 2009)
	qE quenching site	LHCII proteins	LHCSR proteins	(Bonente et al., 2011; Correa-Galvis, Poschmann, et al., 2016)
	qT	Minor contribution to NPQ; mediated by STN7	Transfer of up to 80% of LHCII; mediated by STT7	(Shang et al., 2023)
Xanthophyll cycle	**Influence on qE**	Zx influences qE	Lutein instead of Zx influences qE	(Niyogi et al., 1997)
	VDE	Luminal VDE, dependent on ascorbate	Atypical stromal CVDE, independent of ascorbate	(Z. Li et al., 2016)

Linear electron flow

Algae represent the largest photosynthetically active organism group world-wide, accounting for almost 50 % of all oxygen production on earth. *C. reinhardtii* possesses only one large chloroplast that contains its own genome, which encodes part of the genetic information of the photosynthetic subunits. Embedded in the chloroplast stroma, multiple thylakoids stack to form appressed regions and non-appressed regions of thylakoid membranes. Thylakoid membranes enclose the thylakoid lumen and are the site of the light reactions (Fig. 2; for review see (Arnon, 1971; Hallenbeck et al., 2018; Leister, 2023)), while the process of carbon fixation is taking place in the pyrenoid and the chloroplast stroma (for review see (Gurrieri et al., 2021; Moroney & Ynalvez, 2007)). The light reactions are catalyzed by three large protein supercomplexes in the thylakoid membrane, photosystem II (PSII), cytochrome b_6f (Cyt b_6f) and photosystem I (PSI) which carry out the photosynthetic electron transport form H_2O (at PSII) to $NADP^+$ at PSI (Fig. 2). Coupled to this light-driven electron transport chain is the transfer of protons from the stroma into the thylakoid lumen, which generates the proton motif force (*pmf*) across the thylakoid membrane. The *pmf* is used to drive ATP synthesis by reflux of protons to the stroma through the ATP synthase which finally forms ATP from ADP and P_i in the chloroplast stroma. Hence, this light-driven electron and proton transport leads to the formation of NADPH and ATP, which are then used to drive carbon fixation in the Calvin-Benson-Bassham cycle.

Electron transport is initiated at PSII, where the light-induced electron transfer from the P680 reaction center to the primary quinone acceptor Q_A induces the oxidation of water, where H_2O is split into electrons, protons and molecular oxygen. A more detailed look at the PSII structure and function is provided in the next section. Electrons are transferred inside of PSII from the tightly bound quinone Q_A to the loosely bound Q_B and by that to the plastoquinone (PQ) pool. After reduction and protonation, the formed plastoquinol (PQH_2) transfers the electrons to the Cyt b_6f complex. With about 20 ms, PQH_2 oxidation at Cyt b_6f via PQH_2 oxidation is the rate limiting step of photosynthetic electron transport (Dietz & Pfannschmidt, 2011; Stiehl & Witt, 1969). PQH_2 oxidation is further strictly regulated by the thylakoid lumen pH and is thus the site of photosynthetic control of the electron transport (Foyer et al., 1990; D. M. Kramer et al., 1999). Cyt b_6f is a homodimer consisting of eight subunits per monomer (Stroebel et al., 2003). Cyt b_6f pumps another two protons per electron from the stroma into the lumen while transferring an electron to plastocyanin which is a mobile electron carrier protein at the luminal side of the thylakoid membrane (Redinbo et al., 1993). From plastocyanin, electrons are channeled to PSI. The monomeric PSI complex is a multisubunit protein supercomplex with multiple attached light-harvesting complex (LHC) proteins. The PSI complex has two rings of four LHCI proteins arranged between subunits PsaG and PsaH (Kargul et al., 2003). In

addition, cryo-EM showed that two more LHCI proteins bind close to the core at the other side of the complex (Su et al., 2019; Suga et al., 2019). Therefore, the antenna complex of *C. reinhard*tii at PSI is larger than in higher plants where only four LHCI proteins are bound to PSI. Light-induced charge separation in PSI enforces electron transfer from plastocyanin to ferredoxin (Fd) via the P700 reaction center. The ferredoxin-NADP reductase (FNR) reduces $NADP^+$ to NADPH which is subsequently used for carbon fixation.

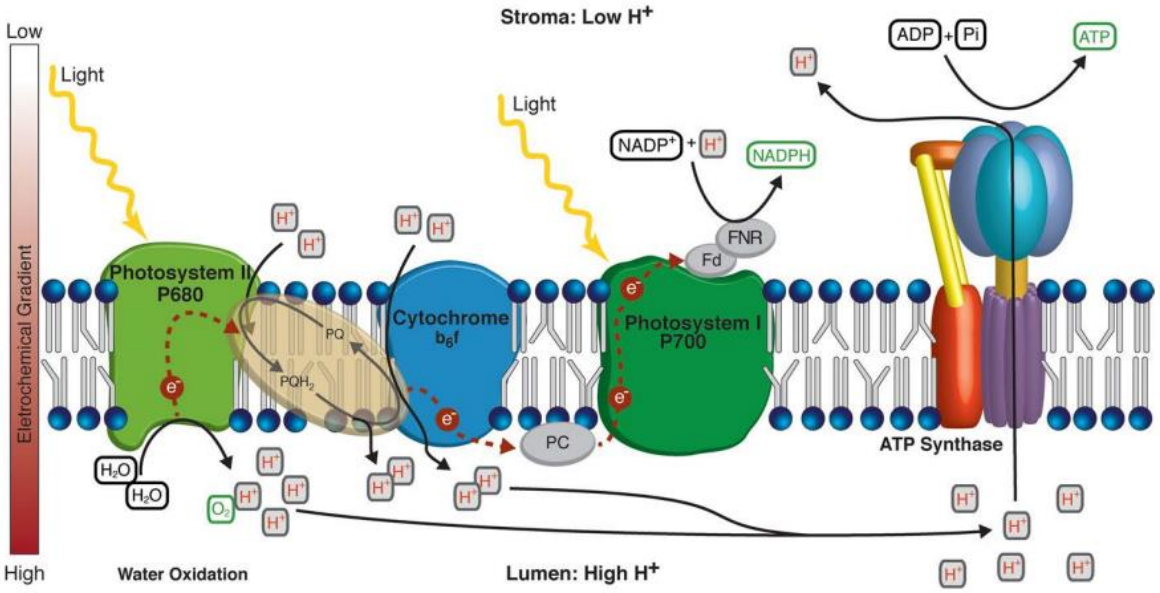

Figure 2: Light reactions in photosynthesis. From (Hallenbeck et al., 2018)

Photosystem II

PSII is a large protein supercomplex that catalyzes the light-induced splitting of water to transfer electrons to plastoquinone. PSII is a homodimer consisting of a heterodimeric core of D1 and D2 (PsbA and PsbD), which is surrounded by the core antenna proteins CP47 and CP43 (PsbB and PsbC) (Burton-Smith et al., 2019) (Fig. 3). Close to the core, PsbE and PsbF form cytochrome b_{559}. Further subunits forming the intramembrane PSII are PsbH, PsbI, PsbJ, PsbK, PsbL,PsbM, PsbR, PsbT, PsbW, PsbX, PsbZ and Ycf12 (Sheng et al., 2019). PsbO, PsbP and PsbQ are extrinsic subunits located at the luminal side. PsbL and PsbJ regulate electron flow to the plastoquinone pool (Ohad et al., 2004). PsbL together with PsbM and PsbT is involved in the dimerization of PSII (Fagerlund et al., 2020). For efficient light harvesting and to channel excitation energy to the PSII core, PSII possesses two monomeric LHC proteins CP26 and CP29, which are associated with the core antenna proteins CP43 and CP47, respectively (Sheng et al., 2019). In addition, in total six trimeric LHCII complexes bind to the PSII homodimer to ensure efficient light-harvesting. These LHCII trimers are either strongly

(S), moderately (M) or loosely (L) bound to the PSII core (C), giving rise to the so-called $C_2S_2M_2L_2$ structure of the PSII homodimer (Fig. 3). Hence, each reaction center core in the homodimeric structure is associated with three LHC trimers and two LHC monomers. The main task of LHC proteins is to harvest light to channel excitation energy efficiently to the PSII core. LHC proteins bind 4 carotenoids and 14 chlorophylls (Chl), the latter consisting of eight Chl a and six Chl b (for review see (Croce & van Amerongen, 2020)). This is in contrast with the PSII core where only Chl a is located.

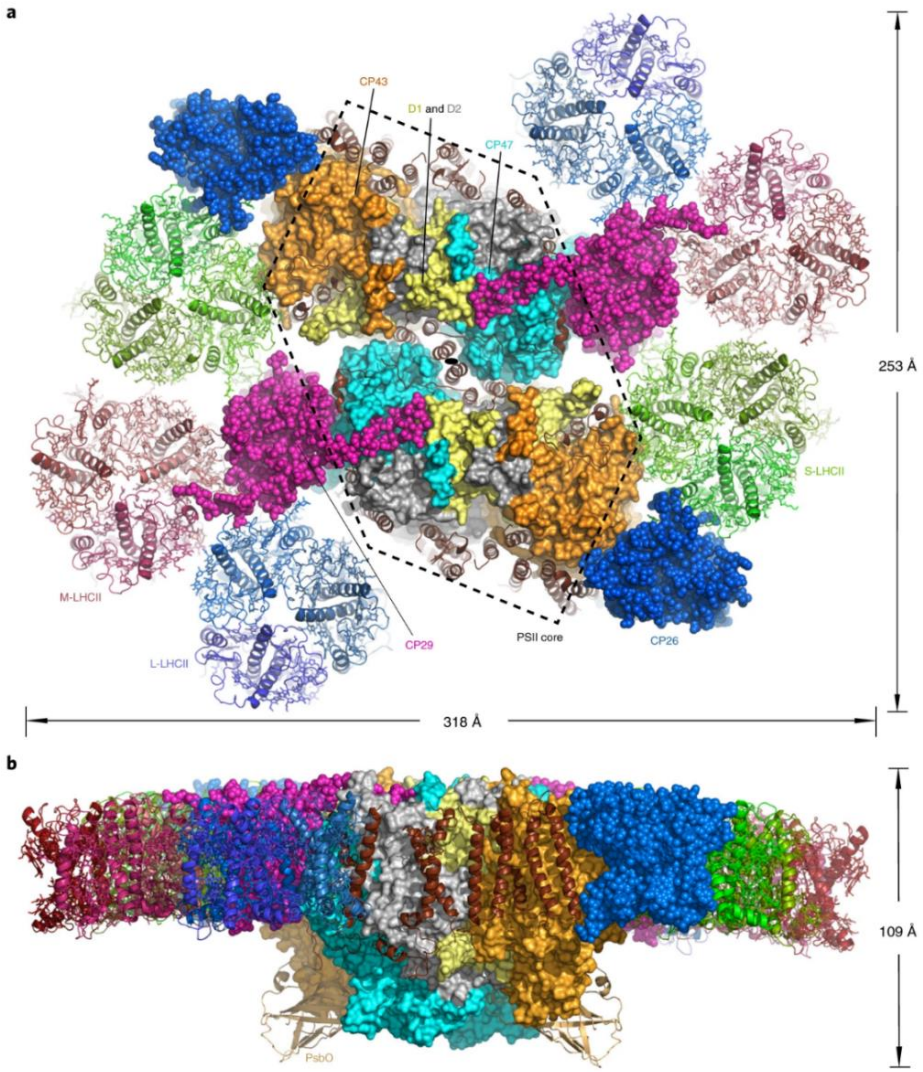

Figure 3: Structure of PSII-LHCII supercomplex. (A) Homodimeric PSII-LHCII supercomplex as seen from the stromal side. Inside the dashed line, the PSII core elements are located. CP26 and CP29 are bound tightly to the PSII core. Three trimeric LHCII proteins are bound per monomer either loosely (L), moderately (M) or strongly (S). (B) Side view of the PSII-LHCII supercomplex along the thylakoid membrane with the stromal side on the top. Figure from (Sheng et al., 2019).

Cyclic electron flow

Photosynthetic organisms mostly grow photoautotrophically with CO_2 as carbon source. Carbon fixation requires energy (in form of ATP) and reducing power (in form of NADPH) for the reduction of CO_2 to the level of carbohydrates (for review see (Gurrieri et al., 2021; Moroney & Ynalvez, 2007)). ATP is synthesized by the ATP synthase which consists of two units, the membrane anchoring F_0 and the hydrophilic F_1, which is the site of ATP synthesis (Lemaire & Wollman, 1989). As an ion channel enzyme, ATP synthase uses the electrochemical gradient across the thylakoid membrane that is built up by the translocation of protons from the stroma to the thylakoid lumen along with photosynthetic electron transport. In linear electron flow, ATP and NADPH are synthesized in a ratio of about 2.6:2 (Lucker & Kramer, 2013). For carbon fixation, however, ATP and NADPH are needed in a ratio of 3:2. Therefore, aside from the linear electron flow from water to NADPH, most photosynthetic organisms have the ability to perform cyclic electron flow. Cyclic electron flow redirects the electrons from ferredoxin back to the PQ pool and not to $NADP^+$ (Joliot & Johnson, 2011). This process produces neither O_2 nor NADPH but allows for additional ATP production via acidification of the lumen. The fine-tuning and balancing of linear and cyclic electron transfer in photosynthesis is not only important in carbon fixation, but also central under stressful conditions (M. Kramer et al., 2021).

Photo-oxidative stress and NPQ

Excessive light is one of the most common stresses photosynthetic organisms have to cope with. Throughout the day, photosynthetic organisms experience light intensities that often vary rapidly in order of magnitude. Therefore, photosynthetic organisms must be able to switch rapidly (seconds to minutes) between a light harvesting and an energy dissipative state. These acclimations need to be fine-tuned as permanent light avoidance impairs cell growth. However, photosynthetic organisms must not only be able to use very low light intensities efficiently but need to tolerate high light intensities as well. Due to the limited mobility of photosynthetic organisms and their actual need for light consumption, plants and algae are especially prone to photooxidative damage under high light conditions. Excess light energy, i.e. light energy that is absorbed in the antenna but cannot be used for photosynthesis, can lead to the formation of reactive oxygen species (ROS) which include singlet oxygen $^1O_2$, superoxide radical O_2^-, H_2O_2 and hydroxyl radicals HO^- (for review see (Barber & Andersson, 1992; Krieger-Liszkay, 2005)). ROS are predominantly formed in organelles with high oxidative power like the chloroplast. In the chloroplast, ROS are generated either by energy transfer from Chl to O_2 or by electron transfer to O_2 from chlorophylls to O_2. Aside from preventing the formation of ROS, formed ROS can be scavenged by enzymes like superoxide dismutase or catalase. When the

available light exceeds the energy that can be used for photosynthesis, the photosynthetic apparatus gets over-excited. The plastoquinone pool cannot take up further electrons, leading to an increased formation of long-lived excited triplet Chl ($^3Chl^*$) which can rapidly transfer the energy to O_2, resulting in the formation of $^1O_2^*$ (Aro et al., 1993; Foote et al., 1984). On the other hand, electron transfer to O_2 which leads to the formation of superoxide radicals and H_2O_2 is predominantly related to the Mehler reaction at the acceptor side of PSI (Mehler, 1951; Polle, 1996). Photosynthetically derived ROS mostly attack the site of their generation, i.e. the photosystems themselves, but can also harm other proteins, pigments, lipids, or nucleic acids (Di Mascio et al., 2019; Møller et al., 2007). Therefore, photosynthetic organisms require strategies to scavenge ROS in the short and long term (Fig. 4). In the long term, photosynthetic organisms are able to acclimate to prolonged excessive light at different levels, such as by decreasing antenna size (De Marchin et al., 2014), by adjusting leaf thickness (Kishitani & Tsunoda, 1982) or by increasing the anti-oxidative capacity (Erickson et al., 2015). At the level of membrane organization, thylakoid membranes are expected to tightly stack in dark or low light conditions but to swell in high light (Kirchhoff et al., 2011). Additionally, the rate of cyclic electron flow is upregulated in response to high light, leading to photoprotection of PSI by removing excess electrons at the acceptor side of PSI (Joliot & Johnson, 2011). However, the most impactful strategy in response to excess light and in prevention of ROS formation is the activation of non-photochemical quenching (NPQ) of excitation energy. NPQ consists of all potential excitation energy sinks besides photochemical quenching (photosynthesis) or chlorophyll fluorescence emission and can easily be measured by pulse-amplitude modulation (PAM) fluorometry (for review see (Schreiber, 2004)). Since the dynamics of NPQ activation and inactivation in response to fluctuating light conditions may limit photosynthesis, the modification of NPQ properties became a promising tool to improve the photosynthetic efficiency and yield of valuable crops (Kromdijk et al., 2016). To identify possible targets for optimizing NPQ properties, it is crucial to understand the NPQ processes in detail. The overall NPQ response is composed of different components and mechanisms, which differ in their efficiency and molecular site of protection and importantly, in their response time (Fig. 4). The four biggest components of NPQ are termed qE (Erickson et al., 2015), qT (Goldschmidt-Clermont & Bassi, 2015), qZ (Niyogi et al., 1997) and qI (Aro et al., 1993) which are introduced in the following with special emphasis on qZ and qE.

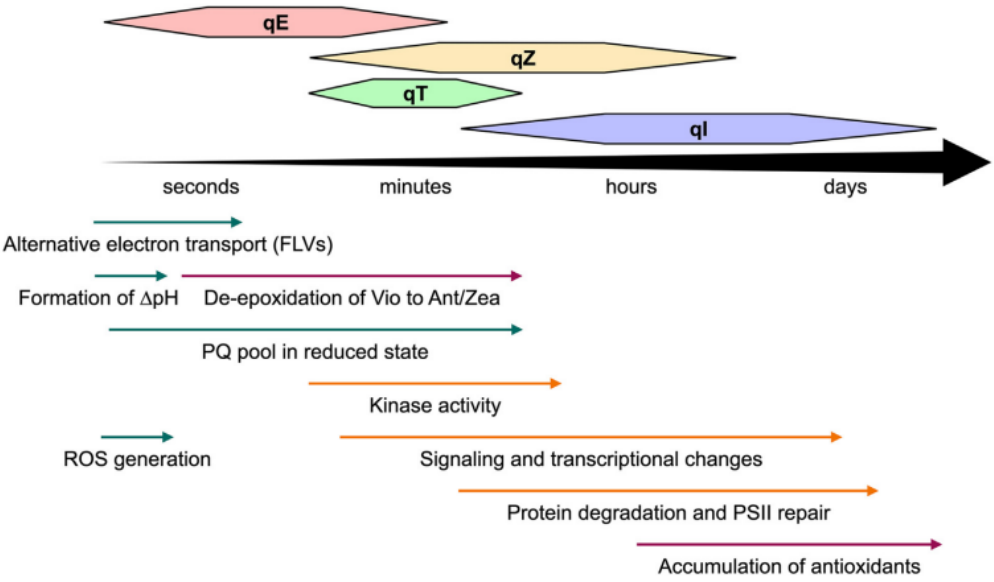

Figure 4: Relative time scales of high light responses. The four major components of NPQ and their relative time frame are shown at the top. Other important factors in acclimation to high light stress are given below, with green arrows indicating a response within seconds, yellow arrows indicating a response within minutes or longer and purple arrows indicating occurrence within a varying time frame. From (Iwai et al., 2023)

Photoinhibition (qI)

As the slowest element of NPQ, photoinhibition or qI occurs within minutes to hours, also depending on the intensity of the HL. qI is the quenching mediated by photo-oxidative damage and subsequent repair of PSII. The controlled repair of impaired PSII is a safety valve to prevent further damage to other components (Aro et al., 1993). The damage-and-repair cycle is less energy consuming than *de novo* synthesis of the entire PSII supercomplex (Keren et al., 1995). The D1 protein subunit of PSII is often the site of photo-oxidative damage. Damaged D1 is subsequently degraded by Ftsh protease and replaced by newly synthesized D1 (Lindahl et al., 2000; Yoshioka et al., 2006). The repair process itself takes place in the stroma lamellae of the thylakoid membrane (Kato et al., 2012).

State transitions (qT)

qT is quenching via state transitions. Upon state transitions, a fraction of LHCII proteins is reversibly phosphorylated and depending on the phosphorylation status can associate either with PSII or PSI. LHCII phosphorylation is mediated by the kinase STT7 (Depège et al., 2003), the dephosphorylation by specific phosphatases (Cariti et al., 2020). That way, a fraction of the excitation energy can be differentially distributed between PSII to PSI. LHCII transfers energy to PSII in the non-phosphorylated state I and to PSI in the phosphorylated state II. As PSII is

more prone to photooxidative damage, the redistribution of excitation energy to PSI in state II reduces the excitation pressure on the electron transport chain and hence the risk of ROS formation. As a trigger, STT7 is activated when the plastoquinone pool is highly reduced (Dumas et al., 2017; Vener et al., 1997). In *C. reinhardtii*, up to 80% of the LHCII proteins can migrate from PSII to PSI to redistribute the excitation energy (Delosme et al., 1996), while in plants, the contribution of qT to NPQ is negligible (Nilkens et al., 2010). The reversion of LHCII migration to PSI is mediated by phosphatases which have been recently characterized in *C. reinhardtii* (Cariti et al., 2020). State transitions have an enhanced role under fluctuating light conditions as the transition from state I to state II takes place even in dark periods (Steen et al., 2022). qT occurs within minutes after HL initiation and acts as a quick response to HL.

Zeaxanthin-dependent quenching

In plants, the carotenoid zeaxanthin (Zx) fulfils multiple critical functions in the defense against HL stress. As free pigment, Zx can scavenge ROS in the thylakoid membrane (Havaux et al., 2007). Bound to LHCII, Zx is involved in the deactivation of triplet chlorophyll (Pogson & Rissler, 2000). This quenching mechanism is known as qZ and becomes activated within minutes of high light stress. In plants, Zx is furthermore directly involved in the most impactful component of NPQ, qE (see next chapter) (Kalituho et al., 2007; Nilkens et al., 2010; Troiano et al., 2021). In contrast, Zx has no immediate impact on qE in *C. reinhardtii*, since mutants without the ability to synthesize Zx were not impaired in qE (Niyogi et al., 1997). Instead, lutein appears to have a higher impact on LHCSR3-mediated qE in *C. reinhardtii* (Bonente et al., 2011). The accumulation of Zx requires the conversion of violaxanthin (Vx) to Zx via the intermediate antheraxanthin (Ax) (Fig. 5). The de-epoxidation of Vx to Zx is catalyzed by Vx de-epoxidase (VDE) and the epoxidation of Zx back to Vx by Zx epoxidase (ZEP). In lands plants, the VDE is located in the thylakoid lumen and its activity is strictly regulated by the lumen pH (Hager, 1969; Pfündel & Dilley, 1993). This pH regulation of VDE activity ensures that Zx is only synthesized when the electron transport is light saturated. ZEP is localized in the chloroplast stroma (Schwarz et al., 2015) and reconverts Zx back to Vx under low light or in darkness. While CrZEP is homologous to plant ZEP, *C. reinhardtii* possesses an atypical VDE, which is located in the chloroplast stroma and which is related to cyanobacterial lycopene cyclase CruA (Anwaruzzaman et al., 2004; Z. Li et al., 2016). CrVDE is independent of ascorbate, which is needed as a cofactor in plant VDE, but depends on FAD instead (Z. Li et al., 2016; Vidal-Meireles et al., 2020). In turn, plant ZEP is downregulated in response to increasing HL stress and becomes inactivated under severe HL stress likely by H_2O_2 (Holzmann et al., 2022). In addition, the processes of epoxidation and de-epoxidation require membrane fluidity (Jahns et al., 2009) and are limited by diffusion of the xanthophylls in the

lipid bilayer of the thylakoids (Kirchhoff, 2014). In plants, an overexpression of ZEP indicated that it is particularly the amount of ZEP that is rate-limiting for the conversion of Zx to Vx, while an increased VDE amount has no impact on Vx to Zx conversion (Küster et al., 2023).

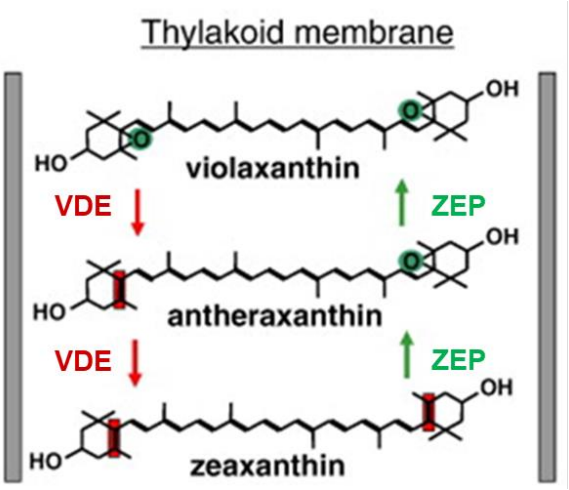

Figure 5: The xanthophyll cycle in the thylakoid membrane. The reactions catalyzed by violaxanthin de-epoxidase (VDE) are indicated by red arrows, the reactions of zeaxanthin epoxidase (ZEP) are indicated by green arrows. Modified from (Jahns et al., 2009)

qE and LHCSR proteins

qE describes the energy-dependent or pH-dependent quenching of excess light energy as heat (Erickson et al., 2015). qE is the fastest component of NPQ that becomes activated or inactivated within seconds to minutes in response to changes in the thylakoid lumen pH and thus in response to changes in the light intensity. Under most conditions, qE is the dominating NPQ process, accounting for the highest portion of NPQ (Krause & Jahns, 2004). In *C. reinhardtii*, qE relies on two light harvesting complex stress related (LHCSR) proteins, LHCSR3 and LHCSR1. Both proteins accumulate right after exposure of *C. reinhardtii* to high light stress, indicating their important role in photoprotection (Peers et al., 2009). The expression level of LHCSR proteins is dependent on the intensity of HL (Redekop et al., 2022). The thylakoid lumen pH has a central function in the activation and regulation of qE. HL-induced lumen acidification is sensed by LHCSR proteins due to protonation of specific amino acids (Bonente et al., 2011; Peers et al., 2009). The luminal acidification is mediated by the increased photosynthetic rate but also by upregulated cyclic electron flow (Joliot & Johnson, 2011). LHCSR proteins bind pigments themselves and are the actual site of light quenching (Perozeni et al., 2020). LHCSR proteins also interact with LHCII proteins and thus reorganize the PSII antenna (Bonente et al., 2011). That way, less excitation energy reaches the PSII reaction center, which reduces the excitation pressure and by that the formation of harmful ROS. For

efficient migration of LHCII proteins, it is especially important to regulate membrane fluidity locally around PSII-LHCII complexes (Yamamoto et al., 2013). In contrast to higher plants, where Zx has a large influence on qE, qE is rather unaffected by Zx levels in *C. reinhardtii* (Niyogi et al., 1997). Even though binding of Zx to LHCSR3 has been observed (Troiano et al., 2021), its contribution to LHCSR3-mediated qE appears to be negligible (Bonente et al., 2011; Tian et al., 2019). Initially, the three LHCSR genes, namely *LHCSR1*, *LHCSR3.1* and *LHCSR3.2,* were found to be expressed in response to high light exposure (Peers et al., 2009). Later, however, it was shown that other stress factors can induce LHCSR expression, too. LHCSR3 gene expression has been triggered by blue light (Petroutsos et al., 2016) as well as by CO_2 deprivation (Águila Ruiz-Sola et al., 2023; Miura et al., 2004; Redekop et al., 2022), sulfur deprivation (Zhang et al., 2004) or iron deprivation (Naumann et al., 2007). LHCSR1 gene expression was additionally inducible by UV light (Allorent et al., 2016).

While the three LHCSR genes differ in only very few nucleotides, LHCSR3 and LHCSR1 have quite distinct functional properties. LHCSR3 is the pH sensor with the highest impact on qE as knock-out mutants of LHCSR3 are severely impaired in qE induction (Peers et al., 2009). LHCSR3 is most likely also the site of quenching by binding to LHCII proteins. LHCSR3 binds 7-8 chlorophylls and 2-3 carotenoids (Troiano et al., 2021). Interaction of LHCSR1 and LHCII proteins has been observed as well (Dinc et al., 2016). Generally, LHCSR1 knock-out mutants were only slightly hampered in qE induction (Peers et al., 2009), but LHCSR1 has been linked to have an impact on the distribution of excitation energy from PSII to PSI (Kosuge et al., 2018). When all LHCSR genes are knocked out, cells lose the ability to activate qE entirely (Kosuge et al., 2018) and are prone to increased generation of $^1O_2$ (Perozeni et al., 2018). As potential interaction partners of LHCSR proteins, LHCBM1 (Elrad et al., 2002), PsbR (Xue et al., 2015) or antenna complexes CP26 and CP29 (Cazzaniga et al., 2020) have been suggested. Fittingly, in a low-resolution single-particle electron microscopy study, a monomeric CP26 and a trimeric LHCII were characterized as LHCSR3 binding spots (Semchonok et al., 2017). Due to their central role in qE quenching, LHCSR proteins are of high importance in light stress defense in *C. reinhardtii* and other green algae. Interestingly, LHCSR proteins got lost during evolution to modern higher plants which do not possess LHCSR genes but use a different protein as the main pH sensor, the PsbS protein.

PsbS

In higher plants, PsbS acts as prime pH sensor and qE regulator (X. P. Li et al., 2000) similar to LHCSR3 in *C. reinhardtii.* The PsbS gene is expressed constitutively but *PSBS* expression is increased when plants are subject to high light stress (Zones et al., 2015). PsbS in higher plants is activated by protonation of two lumen-exposed glutamate residues (X. P. Li et al., 2004). Protonation leads to the monomerization of PsbS dimers which subsequently recruit LHCII trimers to quench light (Fig. 6; (Bergantino et al., 2003; Correa-Galvis, Poschmann, et al., 2016)). In contrast to LHCSR proteins, PsbS does not bind any pigments (Bonente et al., 2008) and PsbS is thus unable to act as quencher of excitation energy itself. The observation, that full qE capacity can be restored in a PsbS-less mutant by an artificial ΔpH rise, supports the view that PsbS is not a quencher itself (Johnson & Ruban, 2011).

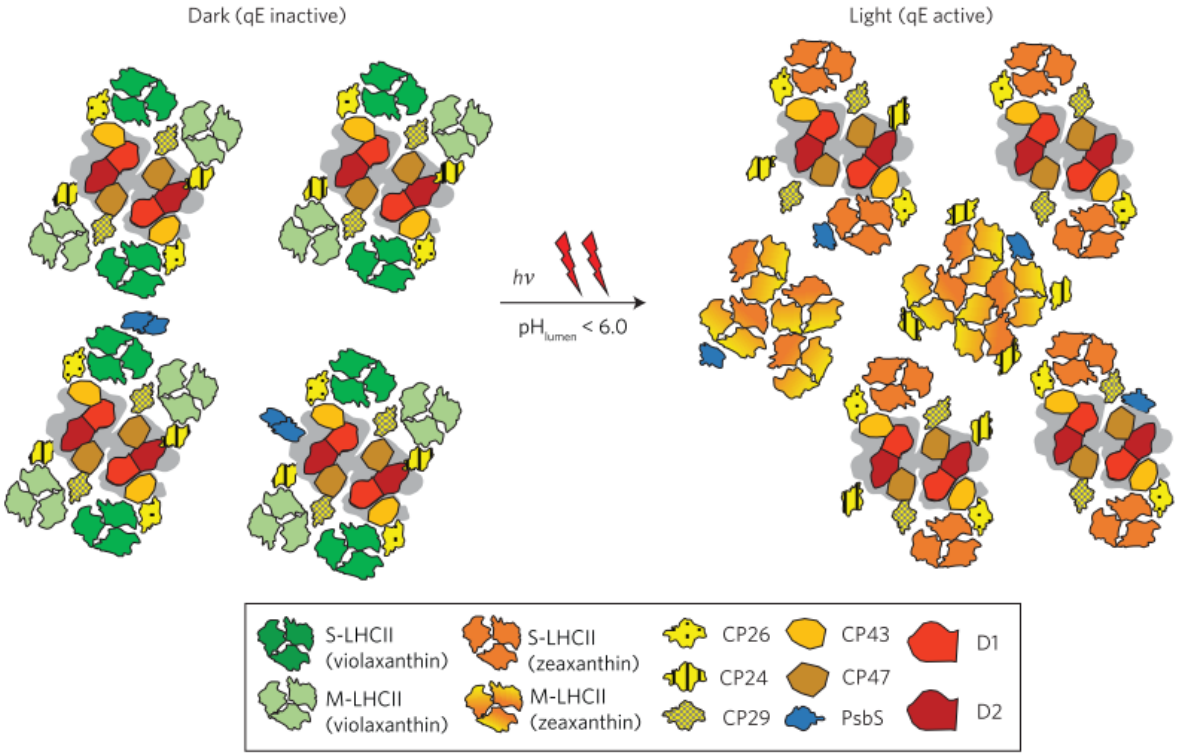

Figure 6: Model for PsbS-induced conformational changes in PSII-LHCII supercomplex of *Arabidopsis thaliana*. In the dark, the PSII-LHCII supercomplexes are in a light harvesting state. In light stress conditions, the lumen acidifies which leads to numerous changes. PsbS is supposed to be monomerized by the pH shift and attaches to either free LHCII aggregates or PSII-bound LHCIIs, forming two potential quenching sites. The pH shift activates violaxanthin de-epoxidase which catalyzes the conversion of violaxanthin to zeaxanthin. From: (Correa-Galvis, Poschmann, et al., 2016)

In contrast to vascular plants, ancient photosynthetic organisms used LHCSR proteins as qE regulators. Plants lost the *LHCSR* genes and replaced some of the functional properties of

LHCSR proteins by PsbS. Organisms containing both LHCSR and PsbS genes offer insight into the evolution of photoprotection. The moss *Physcomitrella patens* contains both genes and both influence NPQ and qE capacity (Gerotto et al., 2011). Green algae like *C. reinhardtii* possess both LHCSR and PsbS genes, too, but while the function and regulation of LHCSR proteins is well understood, the role of PsbS is still under debate. *C. reinhardtii* possesses two PsbS genes (*PSBS1*, *PSBS2*) which only differ marginally (Anwaruzzaman et al., 2004). CrPsbS and AtPsbS are supposed to have similar structure, and CrPsbS also contains the two lumen exposed glutamate residues (E103 and E208) that are likely to be the pH sensors (Fig. 7).

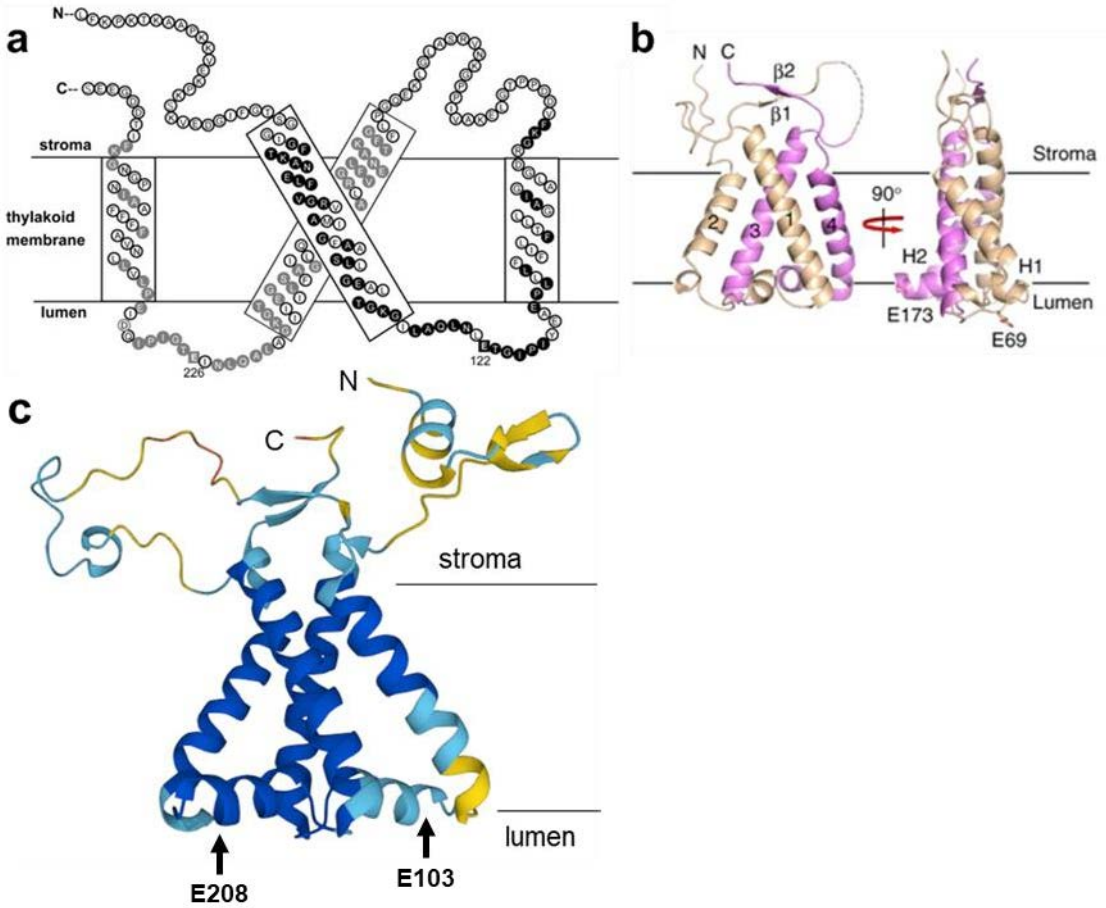

Figure 7: Models for PsbS structures from plants and algae. (a) Schematic view of the plant PsbS structure. The two glutamate residues which have been suggested to sense lumen pH are indicated (here: E122 and E226). Modified after (X. P. Li et al., 2004). (b) PsbS crystal structure in the thylakoid membrane. The two pH sensing glutamate residues are indicated (here: E69 and E173). Modified after (Fan et al., 2015). (c) Predicted structure of PsbS1 of *C. reinhardtii*. The two lumen exposed glutamates (E103, E208) which have been suggested to sense pH are indicated. The prediction of the PsbS structure was performed with AlphaFold (Jumper et al., 2021). The putative chloroplast transport sequence was cut off.

The function of PsbS in *C. reinhardtii* is still subject to speculation. PsbS is not required to establish maximum qE and cannot rescue a knock-out of LHCSR3 (Redekop et al., 2020; Tibiletti et al., 2016). Therefore, a direct involvement of PsbS in qE in *C. reinhardtii* is unlikely. It has been speculated that PsbS is rather involved in thylakoid membrane reorganization enabling efficient and fast establishment of NPQ (Redekop et al., 2020). PsbS might contribute to photoprotection especially in the early stages of high light defense due to its distinct regulation pattern (Correa-Galvis, Redekop, et al., 2016). PsbS accumulates right after a shift from low light to high light but is degraded after only 8 to 14 h of high light (Fig. 8A) (Redekop et al., 2020; Tibiletti et al., 2016). The degradation pattern is specific to CrPsbS and was not observed for *C. reinhardtii* LHCSR proteins (Correa-Galvis, Redekop, et al., 2016) or for plant PsbS (X. P. Li et al., 2004). Up to this point, it is not known whether the degradation is part of the HL defense. The regulatory mechanisms of the degradation of PsbS are also not known yet. It is striking that the degradation occurs even in over-expressing lines (Redekop et al., 2020; Tibiletti et al., 2016). PsbS over-expressing lines have shown a weak phenotype with no alterations in qE, LHCSR accumulation or fitness under constant high light conditions (Fig. 8B). Other environmental factors than high light stress influence PsbS regulation, too. Low CO_2 conditions strongly reduce PsbS degradation (Correa-Galvis, Redekop, et al., 2016), UV-B light triggers PsbS expression to similar levels as high light (Redekop et al., 2022) and nitrogen starvation leads to an enhanced PsbS expression (Miller et al., 2010). In plants, PsbS accumulation is also triggered by long-term drought (Chen et al., 2016). Aside from its function as pH sensor in plants, it has been speculated that PsbS leads to conformational changes in the thylakoid membrane that enable efficient qE by increasing LHCII mobility (Daskalakis et al., 2019; Johnson et al., 2011). In *C. reinhardtii*, the same potential role is conceivable, too (Redekop et al., 2020).

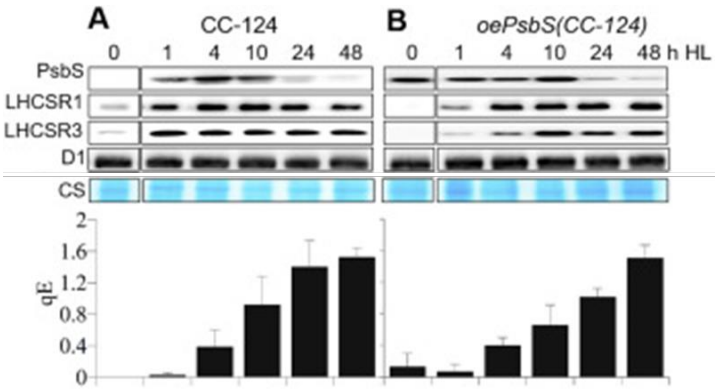

Figure 8: Protein accumulation and qE of wild type cc124 (A) and a PsbS overexpressing line (B) during 48 h of HL acclimation. Upper part: Immunoblot analysis of the accumulation of PsbS, LHCSR1, LHCSR3 and D1 with Coomassie staining (CS) as loading control. In the Western Blots, 40 µg of total protein were loaded for PsbS and 5 µg of total protein were loaded for LHCSR3 and D1 detection onto SDS-PAGEs. Lower part: activation of the qE capacity. In comparison to the wild type, the PsbS over-expressing line shows a weak phenotype regarding qE and the accumulation of LHCSR proteins. Modified after (Redekop et al., 2020)

Thylakoid lipids and membrane fluidity

As mentioned before, the thylakoid membrane fluidity is important for remodeling of the thylakoid membrane during high light stress. An important factor in the functioning of photosynthetic electron transport is the lipid bilayer matrix of the thylakoid membranes, in which the protein complexes of the light reactions are embedded. The thylakoids form appressed (grana in higher plants) and non-appressed regions inside the chloroplast. While PSI and ATP synthase localize to the non-appressed regions, PSII is predominantly present in the appressed regions (Wietrzynski et al., 2020; Yamamoto, 2016). Cyt b_6f is evenly distributed between appressed and non-appressed regions. PsbS localizes to the PSII-rich appressed regions, too (Redekop et al., 2020). It is suggested that PSI is too bulky to fit into the appressed regions (Engel et al., 2015). Under high light stress in plants, the thylakoids reorganize to build larger grana. The two neutral galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) form 50 mol% and 30 mol% of total thylakoid lipids, respectively, while the remainder is constituted of anionic lipids, sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG) (Mendiola-Morgenthaler et al., 1985). The lipid composition can be adjusted to increase or decrease membrane fluidity which is defined as the extent of molecular disorder or movement in the lipid bilayer. It is especially the unsaturation of lipids that increases membrane fluidity (Murata & Los, 1997). In addition, lipids are also integrated in some of the protein complexes involved in the light reactions of photosynthesis such as PSII and PSI (Kern et al., 2009). Regarding photoprotection, it is worth mentioning that NPQ processes are reliant on altered membrane fluidity. Efficient movement

inside of the thylakoid membrane is a prerequisite for any adaptation to stressful conditions (Yamamoto et al., 2014). For example, damaged D1 protein needs to move to the stroma lamellae for repair (Kato et al., 2012), LHCII is reorganized during qE induction (Johnson et al., 2011), and upon state transitions, phosphorylated LHCII moves from PSII to PSI (Depège et al., 2003). The lipid composition and membrane fluidity are further important for the xanthophyll cycle in plants (Jahns et al., 2009). Plant VDE binds especially to MGDG-rich regions which form inverted hexagonal phases (Goss et al., 2007). As described earlier, *C. reinhardtii* possesses an atypical VDE on the stromal side, but the possible role of the membrane lipids for VDE activity is not known yet. Aside from photoinduced changes in membrane fluidity, other environmental factors influence membrane fluidity, too. Thylakoid membrane fluidity was decreased by the addition of oxidants such as Na_2HSO_4 or H_2O_2 (Los & Murata, 2004) or by low temperatures (Barber et al., 1984). In *Saccharomyces cerevisiae*, the membrane fluidity was markedly reduced by hyperosmotic stress (Laroche et al., 2001). It seems thus reasonable to assume that the modulation of the membrane fluidity and especially of the thylakoid membrane fluidity serves important functions during acclimation to different abiotic stressors.

4. AIMS OF THE WORK

The function of PsbS in *C. reinhardtii* is not well understood yet. So far, the regulation of PsbS accumulation and NPQ activation has predominantly been studied under limited, constant light conditions. PsbS is known to accumulate straight after transfer from low light to constant HL, but is degraded after 8-14 h of HL exposure (Tibiletti et al., 2016; Correa-Galvis, Redekop, et al., 2016). It was speculated that PsbS may be at least indirectly involved in qE or NPQ (Correa-Galvis, Redekop, et al., 2016) but later work indicated that a specific involvement of PsbS in NPQ appears unlikely (Redekop et al., 2020). PsbS accumulation also responds to other abiotic factors, such as CO_2 (Correa-Galvis, Redekop, et al., 2016) or nitrogen (Miller et al., 2010). It is thus reasonable to assume that PsbS might be particularly important under either fluctuating HL or under natural light conditions or under other stress conditions. The aim of the present work was to understand the function and regulation of PsbS in *C. reinhardtii* by addressing following aspects:

I. The impact of fluctuating light in combination with intermittent low light or dark phases on cell fitness and on PsbS and NPQ dynamics

II. The impact of combined high light and salt stress on photoprotection and PsbS dynamics

III. Generation and characterization of *PSBS* knock-out strains

5. HYPOTHESIS

Manuscript 1 characterizes the acclimation of *C. reinhardtii* to fluctuating light conditions with special emphasis on PsbS. In detail, accumulation of photoprotective proteins, qE and fitness parameters were analyzed under fluctuating light conditions of high light in alternation with either low light or darkness. Short-day conditions (8 h high light, 16 h low light or dark) and fluctuating light conditions (0.5 h high light, 1 h low light or dark) were compared. Intermittent dark phases led to lower photosynthetic performance and lower energy-dissipation capacity. This went along with an increased pool size of xanthophyll cycle pigments and a reduced degradation of PsbS. Interrupting dark phases are potentially more stressful for *C. reinhardtii* compared to low light phases. Concerning stress parameters, the duration of intermittent low light or darkness phases was less important than actual presence of light. PsbS accumulated to the highest levels in short-day conditions during the high light phase. The degradation was significantly reduced in rhythms with intermittent dark phases.

Manuscript 2 characterizes the photoprotective response of *C. reinhardtii* to combined stress conditions of high light and salinity. In combined stress conditions, *C. reinhardtii* had decreased PS II quantum efficiency. The addition of salt stress did not alter the activation of energy dissipation. The conversion of Vx to Zx was enhanced and PsbS accumulated to significantly higher levels under combined stress conditions. These findings indicate that PsbS and zeaxanthin have a function that is independent of energy-dissipation in the combined stress approach.

Manuscript 3 characterizes PsbS knock-out lines under constant and fluctuating high light conditions. The lack of PsbS did not result in any alternation in energy dissipation when compared to the wild type. This excludes any influence of PsbS on energy-dissipation under the given conditions. In addition, the quantum yield of PSII was not altered in PsbS knock-out mutants. The lack of PsbS reduced the de-epoxidation state of xanthophyll cycle pigments especially in fluctuating light conditions. The function of PsbS is likely not directly linked to energy dissipation. Instead, PsbS might regulate the thylakoid membrane organization and fluidity.

6. MANUSCRIPT 1

Intermittent dark periods negatively affect the photosynthetic performance of *Chlamydomonas reinhardtii* during growth under fluctuating high light

Running title:

Chlamydomonas under fluctuating high light

Fritz Hemker, Nicolas Ammelburger and Peter Jahns*

Photosynthesis and Stress Physiology of Plants, Heinrich-Heine-University Düsseldorf,
40225 Düsseldorf, Germany

*Corresponding author: E-mail, pjahns@hhu.de; Fax, +49-211-8113706

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Abstract

The acclimation of the green algae *Chlamydomoas reinhardtii* to high light has been studied predominantly under continuous illumination of the cells. Here, we investigated the impact of fluctuating high light in alternation with either low light or darkness on photosynthetic performance and on photoprotective responses. Compared to intermittent low light phases, dark phases led to (1) more pronounced reduction of the photosystem II quantum efficiency, (2) reduced degradation of the PsbS protein, (3) lower energy dissipation capacity and (4) an increased pool size of the xanthophyll cycle pigments. These characteristics indicate an increased photo-oxidative stress when high light periods are interrupted by dark phases instead of low light phases. This overall trend was similar when comparing long (8 h) and short (30 min) high light phases being interrupted by long (16 h) and short (60 min) phases of dark or low light, respectively. Only the degradation of PsbS was clearly more efficient during long (16 h) low light phases when compared to short (60 min) low light phases.

1 INTRODUCTION

Chlamydomonas reinhardtii is not only a model organism for plants, but has also emerged as a sustainable alternative for energy production, livestock feed and many other applications. The characterization of optimal growth and cultivation conditions of *Chlamydomonas* is thus of high importance. One central factor that determines the growth properties of *Chlamydomonas* is the growth light regime. Light is not only the ultimate energy source for photosynthesis and thus for biomass production, but may also have damaging properties due to the formation of reactive oxygen species (ROS) which inevitably occurs alongside photosynthetic activity. Like for all photosynthetic organisms, proper acclimation of photosynthetic light utilization in response to different growth light conditions is thus essential for the competitiveness and fitness of *Chlamydomonas* under field and lab conditions. One efficient strategy of plants to reduce ROS formation is the dissipation of excess light as heat, a process also known as non-photochemical quenching (NPQ) of excitation energy. NPQ consists of several components that differ in the underlying quenching mechanisms and act at different time scales (Erickson et al., 2015; Nilkens et al., 2010; Quick & Stitt, 1989; Walters & Horton, 1991). The most rapid one is the pH-regulated qE component which represents the dominating NPQ process under a wide range of conditions (Nilkens et al., 2010). qE is rapidly (within 1-2 min) activated and deactivated in response to the thylakoid lumen pH, which is a reliable indicator of the saturation of photosynthetic electron transport. Precise and rapid regulation of NPQ is required to ensure NPQ is fully active under saturating light conditions but that energy is not dissipated as heat under light limiting conditions (Murchie & Niyogi, 2011; Zhu et al., 2004). Consequently, engineering of plants with optimized NPQ induction and relaxation dynamics was applied to improve photosynthetic efficiency and biomass production (De Souza et al., 2022; Garcia-Molina & Leister, 2020; Kromdijk et al., 2016; Lehretz et al., 2022). The mechanism of state transitions, also termed qT component of NPQ, regulates the energy distribution between the two photosystems in the time range of about 10 min and is based on the reversible phosphorylation of LHCII proteins by STT7 (Depege et al., 2003). State transitions contribute only marginally to the overall NPQ under saturating light conditions in land plants (Nilkens et al., 2010), but significantly contribute to NPQ in *Chlamydomonas*, where up to 80% of the excitation energy absorbed by LHCII proteins can be transferred to PSI upon high light (HL) exposure (Delosme et al., 1996). In the same time range of 10 min, the zeaxanthin (Zx) dependent quenching, qZ, can be activated. qZ contributes significantly to NPQ under HL in vascular plants (Nilkens et al., 2010), but is supposed to have a smaller impact on NPQ in *Chlamydomonas* under constant HL conditions (Bonente et al., 2011; Tian et al., 2019). The xanthophyll Zx is formed from violaxanthin by the violaxanthin de-epoxidase (VDE). In plants, VDE is localized in the thylakoid lumen and its activity is strictly regulated by the lumen pH (Bratt et al., 1995; Hager & Holocher, 1994). In contrast, *Chlamydomonas* possesses an

atypical VDE unrelated to plant VDE, which is located in the stroma, but also requires the build-up of a pH gradient across the thylakoid membrane for activity (Li et al., 2016). Aside from pH-independent qZ, Zx might play a role in pH-dependent qE by binding to LHCSR3 (Troiano et al., 2021).

In *Chlamydomonas*, the LHCSR3 protein acts as sensor of the lumen pH and as the main driver of qE (Ballottari et al., 2016; Peers et al., 2009). LHCSR3 accumulates rapidly in response to high light (HL) (Peers et al., 2009). Activation of LHCSR3 by low pH is supposed to result in interaction with light harvesting complex (LHCII) antenna from photosystem II (PSII) to dissipate excess excitation energy in PSII (Bonente et al., 2011; Girolomoni et al., 2019). The homologous LHCSR1 protein has fewer quenching abilities but contributes to qE as well, possibly related to interaction with LHCI antenna proteins (Dinc et al., 2016).

While LHCSR proteins are crucial for qE in *Chlamydomonas*, PsbS takes on the task as pH sensor and qE activator in vascular plants (Li et al., 2000; Li et al., 2004). PsbS consists of four transmembrane helices and is, similar to LHCSR3, activated by protonation of two lumen-exposed glutamate residues (Anwaruzzaman et al., 2004). Like LHCSR3 in *Chlamydomonas*, PsbS is expected to interact with LHCII antenna proteins upon activation of qE in vascular plants (Correa-Galvis, Poschmann, et al., 2016; Sacharz et al., 2017). *Chlamydomonas* is known to possess two genes for PsbS synthesis, as well (Merchant et al., 2007), however, the precise role of PsbS in *Chlamydomonas* is not fully understood yet. In the moss *Physcomitrella patens*, which contains LHCSR proteins and PsbS, both proteins have been shown to contribute to NPQ (Furukawa et al., 2019; Gerotto et al., 2011). In the multicellular green alga *Ulva linza*, PSBS and LHCSR were also found to be constitutively expressed, and PsbS accumulated to even higher levels in response to HL exposure than LHCSR (Zhang et al., 2013), suggesting that both proteins contribute to NPQ in this alga as well. In contrast, LHCSR and PsbS are not constitutively expressed in *Chlamydomonas*, but accumulate only in response to HL Strikingly, however, PsbS is only transiently expressed under HL and is degraded after about 10-14 h of HL exposure (Correa-Galvis, Redekop, et al., 2016; Tibiletti et al., 2016). So far, the specific role of PsbS remains unknown. Although PsbS is expressed under high light in parallel with the activation of qE, a direct involvement of PsbS in NPQ was not proven so far (Redekop et al., 2020). In LHCSR-deficient mutants, no qE capacity could be restored during (transient) accumulation of PsbS (Correa-Galvis, Redekop, et al., 2016; Tibiletti et al., 2016). Likewise, overexpression of PsbS did not result in altered NPQ levels (Redekop et al., 2020). In a *PSBS* knock-down mutant, the reduced accumulation of PsbS was accompanied by lower amounts of LHCSR3, which in turn lead to a slight reduction of qE. It was speculated that PsbS might contribute to the establishment of a specific interaction of LHCSR with PSII required for qE during NPQ activation (Correa-Galvis, Redekop, et al., 2016).

The regulation pattern of the PsbS amount in *Chlamydomonas* has been of interest in previous studies, too, as it is strikingly different from other photoprotective proteins, such as LHCSR. PsbS accumulates rapidly during HL acclimation even though it accumulates to substoichiometric levels only (Correa-Galvis, Redekop, et al., 2016). The highest accumulation level is reached after 4-8 h of HL and PsbS is actively degraded after 16-24 h of constant HL. This degradation seems to be mandatory since PsbS is entirely degraded after 24 h even in PsbS overexpressing lines (Redekop et al., 2020). It remains unclear, however, whether this process is part of the HL defense. In contrast, LHCSR proteins are not degraded in constant HL, but only about 24 h after HL exposure (Nawrocki et al., 2019). The distinct regulation of the PsbS amount led to speculations about a potential role of PsbS in the onset of the HL response. This view is supported by other characteristics of PsbS expression. Like LHCSR1 proteins, PsbS expression was found to be stimulated by UV-B even under LL, mediated by the photosensor UVR8 (Allorent et al., 2016). Moreover, PsbS accumulation is strongly dependent on the CO_2 availability. Higher levels and longer stability of PsbS were found under low CO_2 conditions, whereas high CO_2 led to accelerated PsbS degradation (Correa-Galvis, Redekop, et al., 2016), implying that photosynthetic efficiency is central to the regulation of PsbS degradation.

Most of the research on the HL acclimation of *Chlamydomonas* has been performed under constant HL conditions. Only recently, more natural growth conditions, which better reflect physiological conditions, have been applied. Exposure to minutely fluctuating HL light alternating with darkness provided evidence for an interplay of LHCSR-mediated qE and STT7-mediated qT which varied in dependence of the frequency of the HL periods (Steen et al., 2022). HL acclimation during 12 h day / 12 h night rhythm showed that a constitutive NPQ capability is established under these conditions in parallel with a permanent accumulation of PsbS (Nawrocki et al., 2019).

In this work, we investigated the impact of different fluctuating growth light regimes on the photosynthetic performance and photoprotection of *Chlamydomonas*. We compared 8 h HL / 16 h dark rhythm with short periods of fluctuating light (0.5 h HL, 1 h darkness), and further determined the impact of an intermittent dark phase in comparison with an intermittent low light (LL) phase. Our data provide evidence that prolonged periods of HL exposure, as under SD conditions, are more stressful to the cells than more frequent, but shorter HL periods, as given under FL conditions. Compared to LL periods, intermittent dark periods increase the photoprotective demand for the cells and suppress the degradation of PsbS and Zx epoxidation.

2 MATERIAL AND METHODS

2.1 Cells and growth conditions

The *C. reinhardtii* WT strain cc124 was used for all experiments. Cells were grown at room temperature in TAP medium (heterotrophic growth with acetate as carbon source) for four days. Subsequently, the cultures were transferred to HS medium (HSM, photoautotrophic growth) in 85 mL glass tubes in the Multi-Cultivator MC 1000-OD-MULTI (Photon Systems Instruments, Drásov, Czech Republic) and incubated under constant bubbling with sterilized air for three days in LL. Light regimes were defined as low light (LL; 75 µmol photons m^{-2} s^{-1}) and high light (HL; 800 µmol photons m^{-2} s^{-1}).

After three days in LL, the first sample was taken (day 1, 0 h) and subsequently the HL rhythms were started. Short day (SD) was defined as 8 h of HL and 16 h of LL or dark. Fluctuating light (FL) was defined as 0.5 h of HL and 1 h of LL or dark, so that the total amount of light per day was the same under SD and FL conditions. Cells were analyzed over a total period of 80 h corresponding to four light and three dark / LL periods under SD conditions, and to 49 HL and 48 dark / LL periods under FL conditions (Figure 1). Samples were taken for all growth conditions at the same time, either at the end of a light phase (after 8, 32, 56 and 80 h) or at the end of the dark / LL phase (after 24, 48 and 72 h).

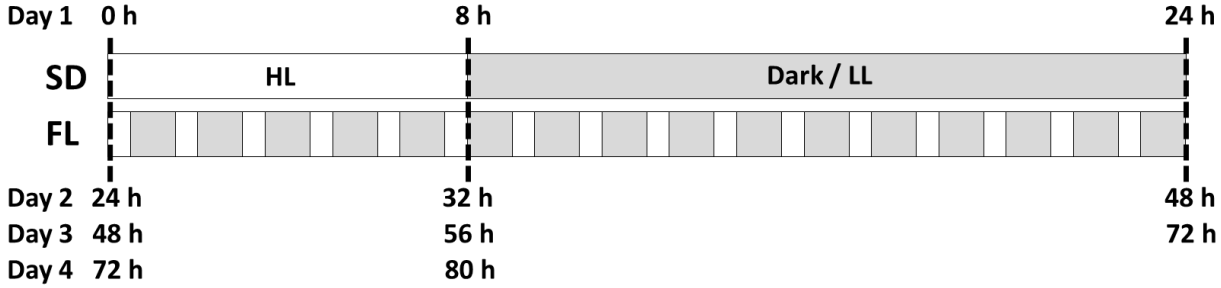

Figure 1. Growth light conditions. The boxes summarize the light regimes for a full day of 24 h. White boxes indicate high light (HL) periods, grey boxes dark or low light (LL) periods. Short day conditions (SD) are defined as 8 h HL and 16 h dark / LL. Fluctuating light (FL) is defined as repetitive sequence of 0.5 h HL and 1 h dark / LL. Dotted lines indicate the time points at which samples were taken, either at the end of a light phase (after 8, 32, 56 and 80 h) or at the end of the dark / LL phase (after 24, 48 and 72 h).

Each sample consisted of 3 mL for chlorophyll fluorescence measurements, 800 µL for pigment analysis and 7 mL for immunoblotting. For immunoblot analyses, additional samples were taken after 4, 28, 52, 76 h. After 8 h and 32 h, the cultures were refilled with the same amount of HSM that has been removed before, to avoid cultures reaching the stationary phase at any point during the experiment.

2.2 Chlorophyll fluorescence

Chlorophyll fluorescence analyses were performed with a Fiber-PAM (Walz, Effeltrich, Germany). For that, 3 mL sample volume was dark adapted for 25 min under continuous shaking at 100 rpm and then applied onto glass fibre filter (Pall, Dreieich, Germany). The filter was fixed in a leaf cuvette and exposed to 1 min of far-red light (about 200 µmol photons m^{-2} s^{-1}) followed by 10 min of red actinic high light (about 1,800 µmol photons m^{-2} s^{-1}) and an additional 5 min dark phase. Saturation pulses (200 ms duration) of 10,000 µmol photons m^{-2} s^{-1} were applied every minute throughout the whole experiment. NPQ was calculated as (Fm/Fm') - 1, with Fm and Fm' being the maximum fluorescence in the dark- and light-adapted state, respectively (Krause & Jahns, 2003).

2.3 SDS-PAGE and Immunoblotting

For protein analysis, 6 mL of culture were harvested and spun down at 2,500 rpm for 5 min. The pellet was resuspended in 150 µL protein extraction buffer (1.6% SDS, 1 M urea, 50 mM Tris/HCl pH 7.6) and stored at -20°C for further analysis. For SDS-PAGE, the protein concentration of each sample was determined using the DC protein assay (Bio-Rad, Feldkirchen, Germany USA). The samples were adjusted in SDS sample buffer to a concentration of 3 mg/mL total protein for PsbS detection and Coomassie staining, and 0.5 mg/mL for LHCSR3, LHCSR1 and PsbA detection as described in (Schwarz et al., 2015). For PsbS gels and Coomassie staining, 30 µg of total protein were loaded on each gel. For LHCSR3, LHCSR1 and PsbA detection, 5 µg of total protein were loaded per sample. Protein separation via SDS-PAGE and immunoblot analysis was performed as described earlier (Kyhse-Andersen, 1984; Laemmli, 1970). PsbS was detected with an antibody specifically designed for *Chlamydomonas* PsbS (Pineda antibody service, Berlin, Germany). LHCSR3 (AS14 2766), LHCSR1 (AS14 2819) and PsbA (AS05 084) antibodies were obtained from Agrisera (Vännäs, Sweden). Detection was performed using Millipore Immobilon Western HRP substrate (Merck, Darmstadt. Germany). Coomassie staining served as a loading control and was performed for 1 h at constant shaking, the destaining process was performed overnight.

2.4 Pigment analysis

800 µL of culture were spun down, washed two times with ddH_2O and resolved in 1 mL acetone (100%). The pigment extract was spun down again, the supernatant was filtered (0.2 µm pore size) and used for reversed phase HPLC (Färber et al., 1997).

2.5 Statistical analysis

Statistical analyses were performed using student's t-test. Significant differences are indicated by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3 RESULTS

3.1 HL induced reduction of PSII efficiency recovers rapidly in LL but not in darkness

To evaluate the impact of HL under the different light conditions on the photosynthetic efficiency, the HL induced changes of the quantum yield of PSII, Y(II), were analyzed. In response to HL exposure, a drop of Y(II) was determined under all growth conditions (Fig. 2). Under SD conditions, nearly full recovery of Y(II) was found during intermittent LL phases, whereas the recovery during corresponding dark phases was less pronounced, though still visible (Fig. 2a). Under FL conditions, however, recovery of Y(II) was only detectable during intermittent LL phases, while Y(II) remained unchanged in intermittent dark phases (Fig. 2b). Comparing the changes during the four days of HL exposure, the pattern of reduction (in HL phases) and recovery (in LL or dark phases) of Y(II) was similar during the whole experiment in FL samples. Under SD conditions, however, Y(II) was gradually reduced with each passing day in dark samples, but not in LL samples (Fig. 2a). Hence, intermittent dark phases had a negative impact on the recovery of HL induced reduction of Y(II) under both FL and SD conditions. In contrast, similar and nearly full recovery of Y(II) was found in samples with intermittent LL phases for FL and SD conditions, indicating that full recovery of Y(II) proceeds rapidly within 1 h of LL. Consequently, also the HL induced reduction of Y(II) occurred fast within 30 min of HL exposure, as can be derived from the samples grown under FL conditions. Therefore, 8 h continuous HL exposure under SD conditions had a similar impact on Y(II) reduction compared to a total HL exposure of 3 h under FL conditions, as reflected by the samples analyzed after 8 h (Fig. 1). In the further course of the experiment, however, 8 h continuous HL phases became more critical than FL HL phases, but only in cells that were exposed to intermittent dark phases (Fig. 2). These findings and interpretations are also reflected by the changes in the cell density during the 4 days of the experiment, as can be derived from changes of the optical density at 680 nm (ΔOD_{680}, Fig. S1), supporting that growth of cells with intermittent dark phases is strongly limited compared to the corresponding growth with intermittent LL phase.

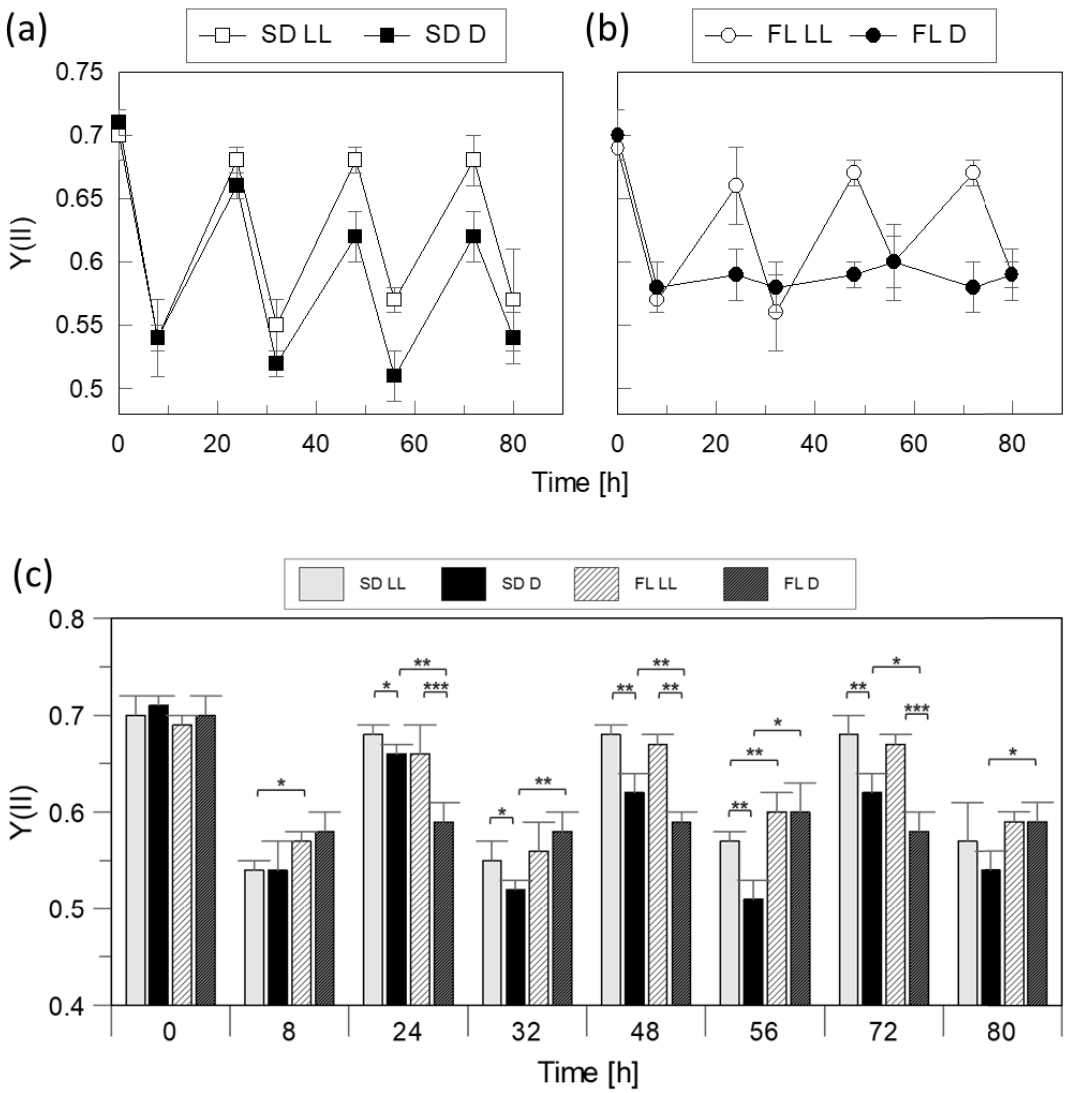

Figure 2. Photosystem II efficiency. The quantum efficiency of PSII, Y(II), was derived from chlorophyll fluorescence analyses. Cells were grown under the light conditions illustrated in Fig. 1. Samples were taken at indicated time and dark adapted for 25 min before measurement. Mean values of 4 biological replicates (n = 4) ± sd are shown for (a) FL conditions and (b) SD conditions. The bar chart shown in (c) illustrates the comparative statistical analysis of the same data sets. Brackets with asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$,) among the light conditions. FL = fluctuating light, LL = low light, D = dark, SD = Short day.

3.2 Intermittent dark periods lead to lower qE capacity than intermittent low light periods

The activation of photoprotective NPQ mechanisms is a well-known response of *Chlamydomonas* to HL exposure. Under moderate high light, the pH-regulated qE component represents the dominating NPQ process. To understand the impact of FL and intermittent LL or dark periods on the qE dynamics, we determined the qE capacity under the different growth conditions.

Before onset of HL exposure, little or no qE was detectable (Fig. 3, 0 h samples). Under both SD and FL conditions, maximum qE capacity of about 0.8 was established after 48 h in LL samples and after 80 h in dark samples (Fig. 3), indicating that the overall amount of light determines the qE induction rather than the light rhythm.

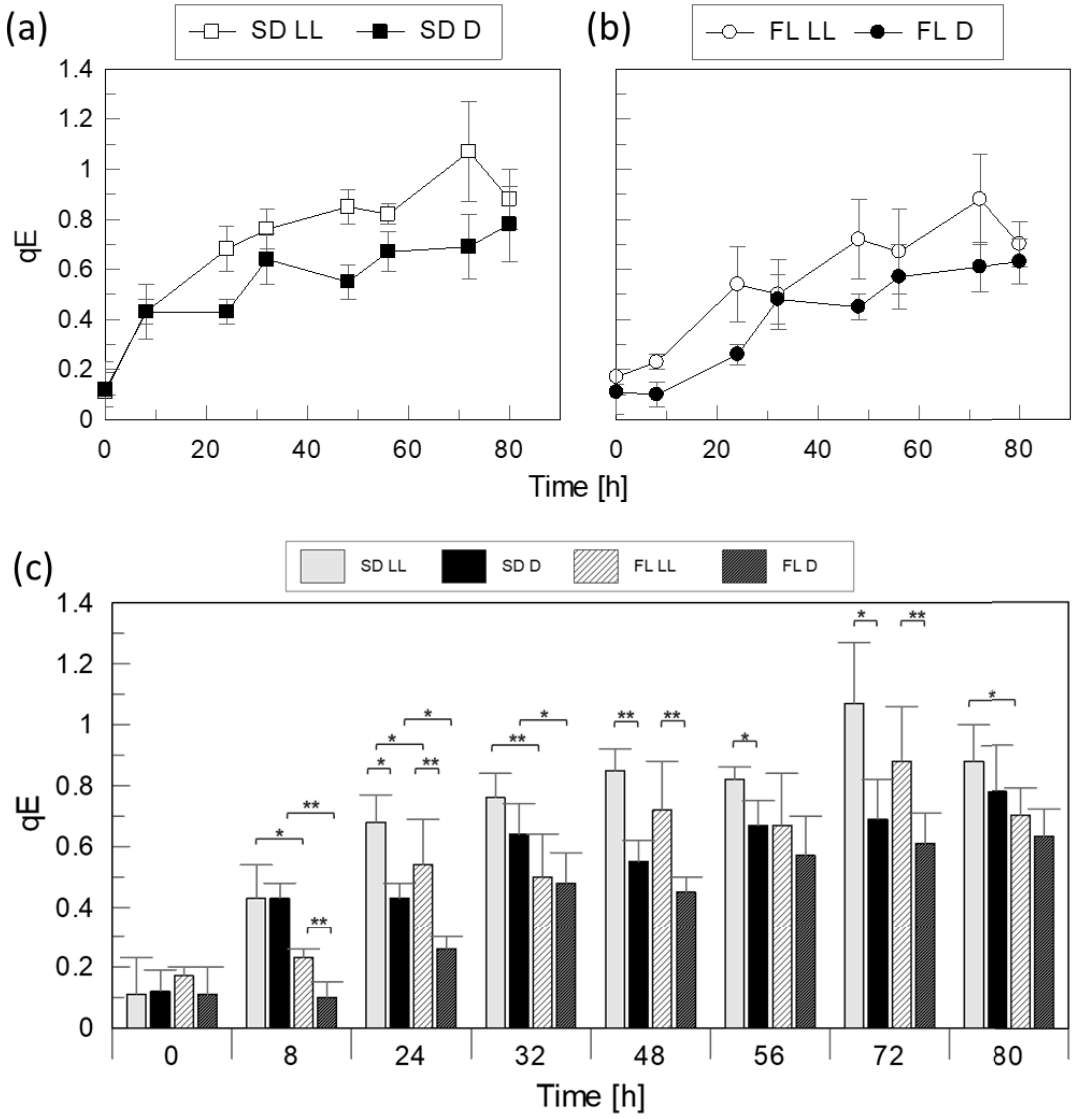

Figure 3. qE capacity. Cells were grown under the light conditions illustrated in Fig. 1. Samples were taken at indicated time and dark adapted for 25 min before measurement. The qE capacity was calculated from the difference of the maximum NPQ at the end of 10 min illumination at 1,800 µmol photons m^{-2} s^{-1} and the NPQ value obtained after subsequent 5 min dark relaxation. Mean values of 4 biological replicates (n = 4) ± sd are shown for (a) SD conditions and (b) FL conditions. The bar chart shown in (c) illustrates the comparative statistical analysis of the same data sets. Brackets with asterisks indicate significant differences (*, $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$,) among the light conditions. FL = fluctuating light, LL = low light, D = dark, SD = Short day.

In line with this notion, the qE capacity increased more rapidly during the first 8 h under SD (Fig. 3a) conditions compared to FL conditions (Fig. 3b), since the cells received only 3 h of HL during the first 8 h growth under FL conditions. Interestingly, the induction of qE capacity was slightly delayed in dark samples compared to LL samples, which was related to an arrest of the qE capacity during intermittent dark periods (Fig. 3). This was most obvious during the 16 h dark period in cells grown under SD conditions (Fig. 3a), but applied in tendency also to FL conditions (Fig. 3b). Therefore, the establishment of a high qE capacity during HL acclimation obviously continues during intermittent LL phases, but not in intermittent dark periods. The difference in the qE capacity of HL / LL and HL / dark samples leveled out after 48 h and 72 h of growth and finally reached similar values after 80 h of HL treatment.

3.3 PsbS degradation is light dependent and strongly delayed in dark periods

The establishment of a high qE capacity in response to HL exposure is known to depend on the expression of LHCSR proteins. Moreover, transient accumulation of PsbS during HL acclimation has been reported. We determined the amount of LHCSR and PsbS proteins in response to the different growth light conditions (Fig. 4). Besides the sampling time points used for the analyses of Y(II) and qE, we took at each day an additional sample 4 h after onset of HL in the SD cycle, since highest PsbS amounts typically accumulate transiently at this time after transfer to HL (Correa-Galvis, Redekop, et al., 2016; Redekop et al., 2020).

LHCSR3 was already detectable in all samples at the beginning of the experiment (Fig. 4a,b). This is at variance with other studies showing that LHCSR3 expression does not occur under LL conditions and can be explained by the rather high intensity of 80 µmol photons $m^{-2}s^{-1}$ defined here as low light. It should be noted, however, that the accumulation of LHCSR3 during LL was not accompanied by the induction of qE (Fig. 3). LHCSR3 levels did not show clear changes during HL exposure under all light regimes (Fig, 3 and Fig S2).

Compared to LHCSR3, only traces of LHCSR1 were detectable at the beginning of the experiment. LHCSR1 accumulated continuously during HL acclimation under all growth conditions and maximum amounts were reached after about 50-60 h of HL acclimation (Fig. 4c,d). Compared to FL conditions, the accumulation of LHCSR1 occurred slightly faster and to higher amounts under SD conditions. However, LHCSR1 accumulation under SD conditions occurred predominantly during the HL periods, but tended to be arrested during intermittent LL or dark phases.

PsbS was not detectable before onset of HL exposure (Fig. 4e,f), supporting the view that PsbS is not expressed under the chosen LL conditions. As expected, PsbS accumulated rapidly upon HL exposure under SD conditions and was degraded in a subsequent LL period.

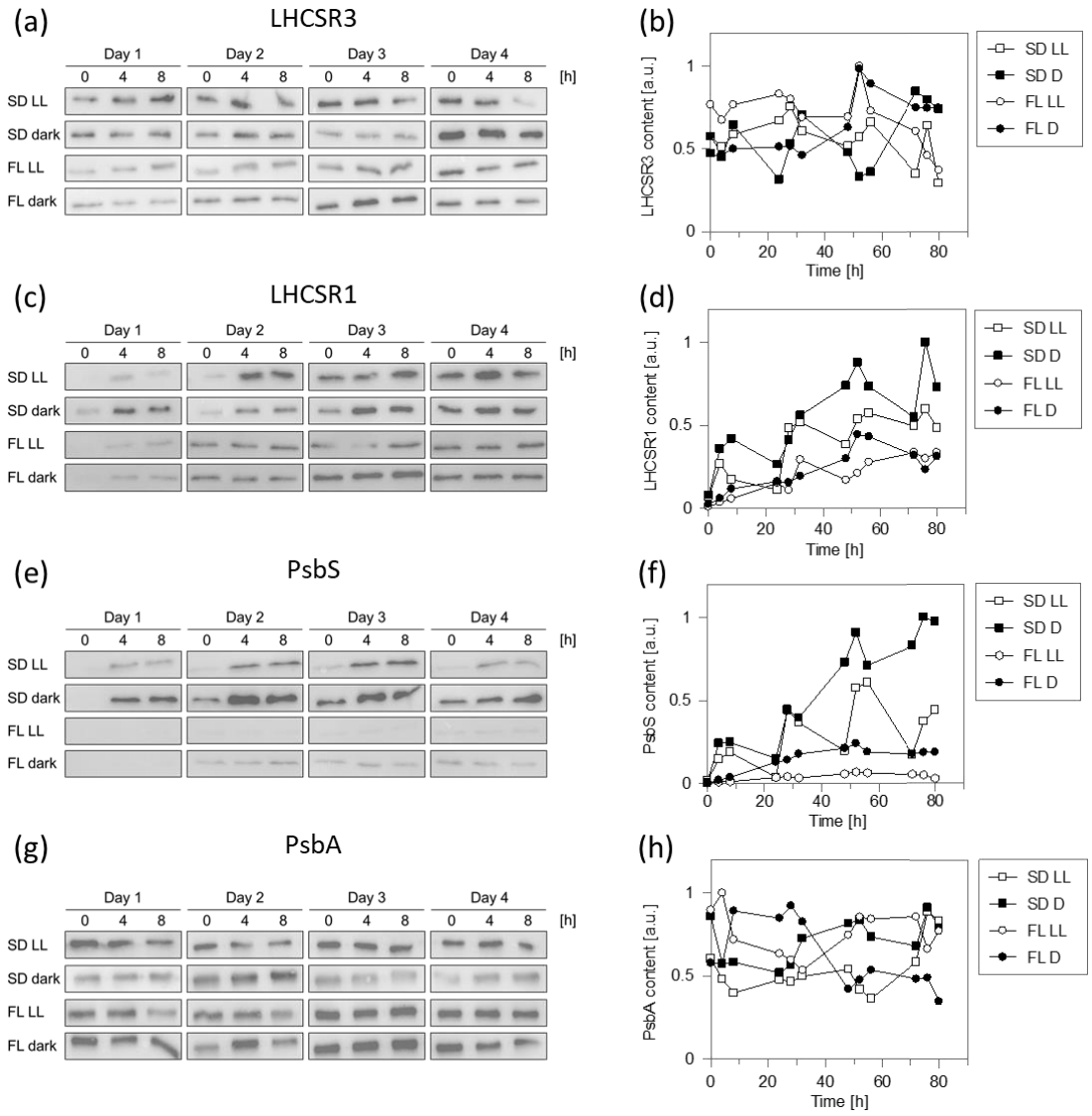

Figure 4. LHCSR and PsbS content. The accumulation of selected proteins was derived from immunoblots. For LHCSR3 (a,b), LHCSR1 (c,d) and PsbA (g,h), 5 µg of total protein were loaded per sample, for PsbS (e,f) 30 µg of total protein was loaded per sample. Representative blots from at least 3 biological replicates are shown on the left side (a,c,e,g). On the right side (b,d,f,h), proteins amount were quantified by densitometric scans of the corresponding blots. For each protein, data were normalized to the maximum determined for each protein. The data represent the mean values from all replicates. For clarity, sd values are not shown, but were typically in the range from 0.1 - 0.5. Statistical analysis of the data, including sd values, is provided in Fig. S2. FL = fluctuating light, LL = low light, D = dark, SD = Short day.

However, PsbS degradation did not occur in darkness. Compared to SD conditions, the accumulation of PsbS was strongly reduced under FL light conditions, particularly with intermittent LL periods. Moreover, no net degradation of PsbS was detectable under FL LL conditions. Under FL dark conditions, pronounced PsbS amounts were detectable after about 24 h, and PsbS accumulated to significantly higher amounts compared to FL LL conditions (Fig. 4f, Fig. S2). Obviously, PsbS accumulation and degradation are light-dependent processes which require longer periods of constant light.

3.4 Intermittent dark periods lead to increased VAZ pool size and delayed Zx epoxidation

We further investigated the impact of different growth light regimes on the de-epoxidation state (DEPS) and on the total amount (VAZ pool size) of the xanthophyll cycle pigments. Both parameters are indicators of HL induced photo-oxidative stress. The initial DEPS values were below 10%, indicating that the chosen LL conditions do not lead to the activation of the Vx de-epoxidase. In cells grown under SD conditions (Fig. 5a), maximum DEPS values of about 60% were reached at the end of all HL periods which were reverted to values of about 10% at the end of the subsequent LL or dark periods. Hence, intermittent LL or dark periods do not alter the response of the conversion of the xanthophyll cycle pigments, at least at the chosen time points. This was different, however, in cells grown under FL conditions (Fig. 5b). Under FL LL conditions, maximum DEPS values of only about 50% were determined at the end of the 0.5 h HL period and the DEPS reverted to about only 30% during the subsequent 1 h LL period., During intermittent dark phases, however, DEPS values remained stable under FL conditions, and the DEPS values at the end of HL period reached highest values of about 60%, as also observed under SD conditions. This indicates that Zx epoxidation is strongly slowed down in darkness, and that 0.5 h of HL is not sufficient to reach maximum DEPS values, even when Zx is only partially reconverted to Vx during the 1 h LL periods under FL conditions.

Interestingly, the VAZ pool size increased in all HL treatments, indicating an increased demand for photoprotection in response to HL. Again, however, clear differences became evident between SD and FL conditions. Under SD conditions, the VAZ pool size per 1000 Chl (a+b) increased from about 50 to about 100 in SD dark cells and to about 80 in SD LL cells. Moreover, a transient reduction of the VAZ pool during the LL and dark phase was found under SD conditions (Fig. 5c). In contrast, the increase of the VAZ pool size was less marked under FL conditions (Fig. 5d), where the VAZ pool size per 1000 Chl (a+b) increased to about 90 in FL dark cells and to about 70 in SD LL cells. Furthermore, no transient reduction of the VAZ pool size was detectable under both FL dark and FL LL conditions. Obviously, the longer continuous HL exposure under SD conditions represents a more severe stress condition than repetitive exposure to shorter HL periods under FL conditions.

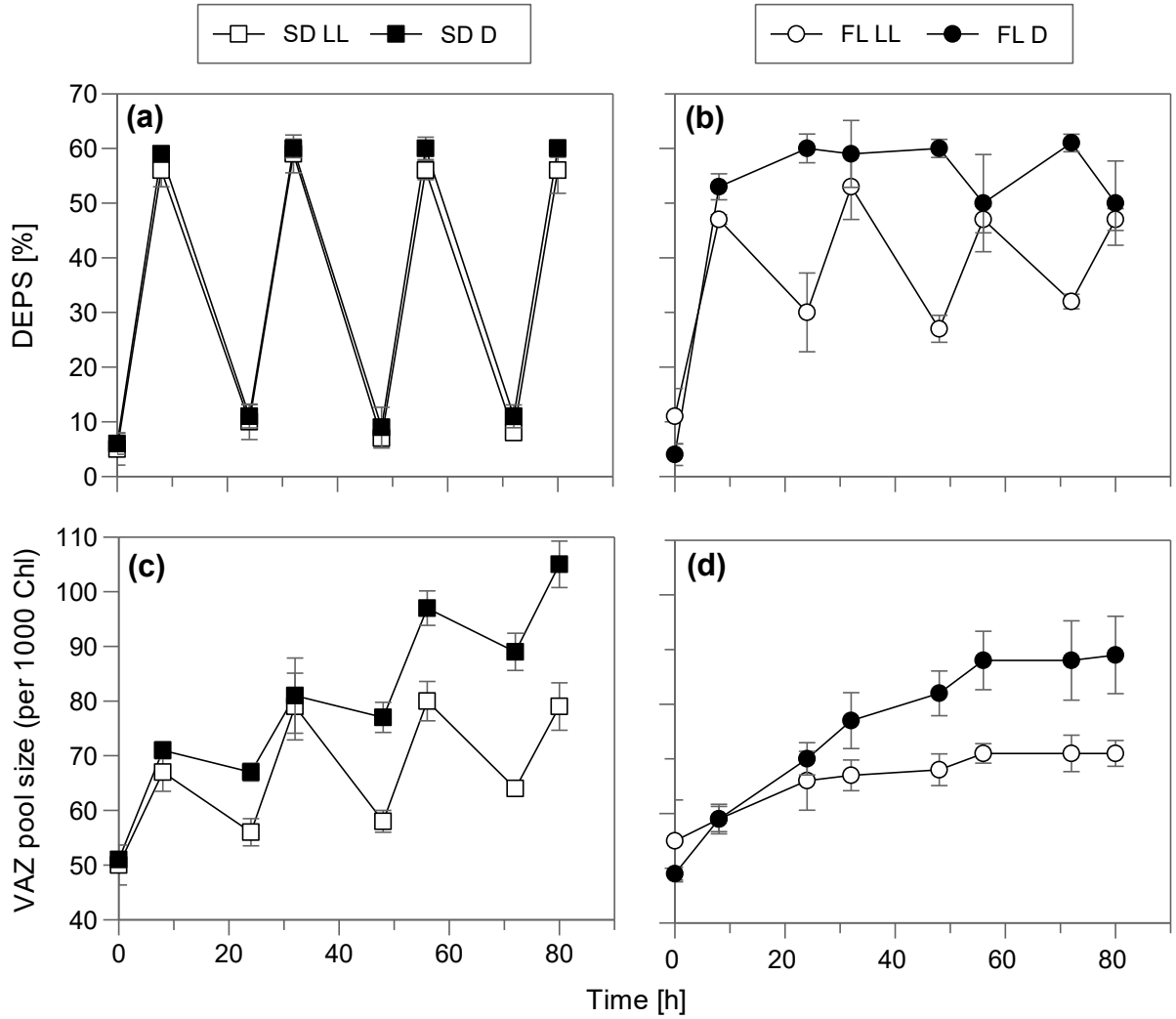

Figure 5. Xanthophyll cycle pigments. Pigments were extracted with acetone and the amounts of the xanthophyll cycle pigments quantified by HPLC analysis. The de-epoxidation state (DEPS) of the xanthophyll cycle pigments violaxanthin (Vx), antheraxanthin (Ax) and zeaxanthin (Zx) is defined as (0.5 Ax + Zx) / (Vx + Ax + Zx)*100, and is shown for cells grown under (a) SD and (b) FL conditions. The sum of the xanthophyll cycle pigments (Vx + Ax + Zx) is defined as VAZ pool size. The VAZ pool size expressed in relation to the chlorophyll (Chl) content (per 1000 Chl a+b) and is shown for cells grown under (c) SD and (d) FL conditions. Cells were grown under the light conditions illustrated in Fig. 1. Mean values of 3 biological replicates (n = 3) ± sd are shown. Statistical analyses are shown in Fig. S3. FL = fluctuating light, LL = low light, D = dark, SD = Short day.

4 DISCUSSION

Proper acclimation of photosynthetic organisms to different growth light intensities is essential for all photosynthetic organisms to ensure efficient photosynthesis. Detailed understanding of the molecular basis of photo-acclimation is required to identify key parameters that may limit growth under both lab and natural conditions. Unicellular algae such as the green algae *Chlamydomonas* are typically cultivated under stress-free conditions under constant light, to allow maximal growth rates and hence biomass production. Likewise, most studies on HL acclimation and photoprotective mechanisms used constant light conditions, which basically differ from natural light conditions. To understand the impact of intermittent dark periods on the regulation and functioning of photoprotective mechanisms, we analyzed key parameters of photoprotection upon HL exposure under SD and FL conditions, each alternating with LL or dark periods.

4.1 The LL intensity differentially affects LHCSR3 expression and qE activation

The intensity of 80 µmol photons $m^{-2}s^{-1}$ used here for LL conditions unraveled different light responses of LHCSR3 expression and qE activation. Whereas significant amounts of LHCSR3 protein accumulated (Fig.4), no qE was detectable at this growth light intensity (Fig. 3). Obviously, the simple presence of LHCSR3 is not sufficient for the induction of qE. It can thus be speculated that specific reorganization of the PSII antenna, such as conformational changes or binding of LHCSR3 to specific sites is required prior to the activation of qE. Since LHCSR3 expression and qE activation are typically induced within a few hours after transfer from LL to HL (Redekop et al., 2020), it can be assumed that a HL induced signal is required for initiation of PSII reorganization, such as the lumen pH or the redox state of specific electron transport components. The LL conditions used here did not lead to the formation of Zx, supporting the view that thylakoid membrane energization and thus lumen acidification are far from being saturated. The HL induced reorganization of PSII might further include synthesis of other photoprotective proteins such as LHCSR1 or PsbS. Expression of these two proteins, was not induced under LL conditions, indicating that the UV-B regulated expression of LHCSR1 and PsbS (Allorent et al., 2016) requires higher light intensities than the UV-B independent expression of LHCSR3. It can thus be concluded that LHCSR3 dependent quenching, which accounts for the major part of qE in *Chlamydomonas*, becomes activated at lower light intensities than LHCSR1 dependent qE (Dinc et al., 2016) and any qE-independent photoprotective functions of PsbS (Redekop et al., 2020).

The establishment of the maximum qE capacity took 48 to 72 h (Fig. 3) and thus similar time as the accumulation of LHCSR1 to maximum amounts (Fig. 4), suggesting that the amount of

LHCSR proteins or LHCSR-dependent reorganization of the PSII antenna may determine the final maximum qE capacity under the respective HL conditions. The increase of the qE capacity was found to be more rapid under SD conditions than under FL conditions, indicating that 8 h of HL applied without intermittent LL or darkness are more efficient in qE activation. Interestingly, the qE capacity increased also during the 16 h LL period under SD conditions, while no further increase was found in corresponding dark periods. Obviously, the molecular processes that determine the increase of the qE capacity depend on energy provided by the light reactions. Possible dissipation of absorbed light energy under light-limited LL conditions is obviously uncritical for *Chlamydomonas*, although LHCSR3 has been shown to form a quenching state at rather high pH of 7.5 (Bonente et al., 2011) and thus likely under LL conditions. The arrest of the qE increase during the 16 h dark period was not accompanied by the reduction of the amount of LHCSR proteins, in agreement with earlier work showing that LHCSR degradation requires more than 24 h after transfer from HL to LL conditions (Nawrocki et al., 2019). Hence, the capability of qE activation is maintained even during 16 h of darkness.

4.2 Intermittent dark periods delay HL acclimation

For all studied parameters, clear differences were observed between intermittent LL and intermittent dark periods under both FL and SD conditions. Compared to LL, dark periods delayed the recovery of HL induced reduction of PSII efficiency (Fig. 2), the generation of a high qE capacity (Fig. 3), the degradation of PsbS (Fig.4), and the increase of the VAZ pool size (Fig. 5). Since all these processes represent typical HL acclimation responses, it can be concluded that intermittent dark phases arrest or strongly delay the process of HL acclimation. Obviously, HL induced acclimation processes do not proceed in dark periods and thus are light-dependent. Taking the establishment of the maximum qE capacity as indicator for HL acclimation, it took about 48 h to reach the HL acclimated state under SD and FL conditions, which corresponds to a total HL exposure time of about 16 h. Compared to growth under continuous HL, where induction of maximum qE capacity took at least 24 h of HL (Redekop et al., 2020), growth under SD and FL conditions seems to require less total HL exposure time for qE induction (and thus HL acclimation) as long as LL and not dark periods alternate with HL. Likely, only a certain HL exposure time is required to trigger the onset of HL acclimation. It would be of interest to determine the threshold of the minimum HL stimulus that is needed to activate the induction of qE and thus HL acclimation.

The negative impact of darkness on HL acclimation might be related to the repeated occurrence of dark to HL transitions, which likely represent the most stressful situation during the applied growth light regimes. Consequently, one would then expect that FL dark conditions are more critical for the fitness of the cells than SD dark conditions. However, this is obviously

not the case, as can be derived from the similar impact of both light regimes on growth (Fig. S1). On the contrary, 8 h of HL in alteration with 16 h dark periods led to stronger inactivation of PSII efficiency (Fig. 2) and to a more pronounced increase of the VAZ pool size (Fig. 5), both indicating increased HL stress under SD dark compared to FL dark conditions. This supports the view that particularly long dark phases have negative impact on HL acclimation.

4.3 The xanthophyll cycle pigment dynamics indicate a critical role of Zx in light utilization

The negative impact of darkness on HL acclimation was also obvious from the dynamics of the DEPS and the size of the VAZ pool (Fig 5). The VAZ pool size is a critical determinant of the amount of Zx accumulating in the thylakoid membrane and an increase of the VAZ pool size is a typical HL response of land plants (Bailey et al., 2004; Demmig-Adams et al., 2012; Mishra et al., 2012; Schumann et al., 2017) and *Chlamydomonas* (Baroli et al., 2003). The more pronounced increase of the VAZ pool size in cells grown with intermittent dark periods indicates an increased HL stress compared to cells grown with intermittent LL periods. The observed transient reduction of the VAZ pool size specifically during long intermittent LL periods under SD conditions (Fig. 5c), might thus reflect the recovery from HL stress under SD LL conditions, in line with the transient reduction of Y(II) (Fig. 2). However, Y(II) also recovered during LL under FL conditions and during long dark periods under SD dark conditions without any impact on the VAZ pool size, indicating a rather specific and dynamic HL acclimation response of the VAZ pool size. Obviously, the VAZ pool size is only decreased after transition from HL to LL and the reduction of the VAZ pool size occurs at slower kinetics than the increase of the VAZ pool size after LL to HL transition. The latter can be particularly concluded from the changes of the VAZ pool size under FL LL conditions, where a continuous increase of the VAZ pool size to similar maximum values as under SD LL conditions was determined. Thus, the increase of the VAZ pool size likely reflects the overall HL exposure time.

The reduction of the VAZ pool size in response to longer LL periods suggests a negative impact of a large VAZ pool, or more precisely of an increased Zx amount, under LL conditions. This might be related to the adverse impact of Zx-dependent energy dissipation under light limited conditions on photosynthetic efficiency. Indeed, the lower Fv/Fm ratio determined in Zx accumulating *Chlamydomonas* mutants (Niyogi et al., 1997; Troiano et al., 2021) suggests a permanent Zx-dependent quenching in presence of high Zx amounts. The similar conclusion can be drawn from our data, when comparing the changes of the DEPS (Fig. 5a,b) with that of the Y(II) (Fig. 2a,b). Strikingly, Y(II) and DEPS showed a clear inversely proportional response: High DEPS correlated under all conditions and at all time points with a low Y(II) and vice versa. This strongly suggests that a high Zx content reduces the photosynthetic efficiency of PSII. In

land plants, a limited photosynthetic efficiency after HL to LL transition has been achieved by acceleration of NPQ relaxation due to overexpression of Zx epoxidase (Garcia-Molina & Leister, 2020; Kromdijk et al., 2016), which leads to accelerated reconversion of Zx to Vx (Küster et al. 2023). Compared to land plants, where Zx serves important functions in qE (Horton et al., 2005; Niyogi et al., 1998), Zx is supposed to have a less prominent role in qE in *Chlamydomonas* (Bonente et al., 2011; Niyogi et al., 1997; Tian et al., 2019). However, recent work provided evidence that binding of Zx to LHCSR3 might contribute to a Zx-dependent portion of qE in *Chlamydomonas* (Troiano et al., 2021), suggesting that Zx binding to LHCSR3 may be critical for the limitation of photosynthetic efficiency after the transition from HL to LL. The parallel increase of the VAZ pool size and of the LHCSR protein content may indicate that the additional pool of VAZ pigments that is formed during HL acclimation of *Chlamydomonas* is bound by LHCSR proteins. However, the decrease of the VAZ pool size specifically during LL under SD conditions without an accompanying decrease of LHCSR proteins seems to contradict this hypothesis. It can thus not be ruled out that the additional VAZ pool formed under HL is localized in the lipid phase of the thylakoid membrane, as has been proposed for vascular plants (Bethmann et al., 2023). Indeed, such a non-protein bound pool of VAZ pigments is supposed to contribute to photoprotection under severe HL stress (Baroli et al., 2003; Bethmann et al., 2019; Havaux & Niyogi, 1999), but does not exclude specific interactions with antenna proteins, such as LHCSR proteins, at the lipid / protein interface.

4.4 The characteristics of PsbS accumulation suggest a role of PsbS under stress conditions

In response to a shift from continuous LL to continuous HL, PsbS protein is known to accumulate transiently within 4 h, but to become degraded actively after about 10 h (Correa-Galvis, Redekop, et al., 2016; Redekop et al., 2020; Tibiletti et al., 2016). The dynamics of PsbS synthesis and degradation are closely related to the photosynthetic light utilization capacity during HL acclimation. Accordingly, an accelerated PsbS degradation was found under high CO_2 (i.e high photosynthetic capacity), while PsbS degradation was delayed under low CO_2 conditions (i.e. low photosynthetic capacity) and in absence of LHCSR proteins, when the capacity of NPQ induction is lacking (Correa-Galvis, Redekop, et al., 2016). Hence, PsbS is particularly accumulated for longer time under conditions when photosynthetic performance is limited either by CO_2 or by lacking NPQ capacity. This suggests that PsbS may be required as long as HL acclimation is not fully completed and implies a photoprotective role of PsbS during HL acclimation, supposedly independent from NPQ (Redekop et al., 2020). This conclusion is fully supported by our data showing that PsbS is accumulating continuously under SD dark conditions, which represent the most stressful growth conditions applied here.

The much lower accumulation of PsbS detectable under FL conditions compared to the corresponding SD conditions (Fig. 4) indicates that prolonged exposure to HL is more stressful to the cells than similar total HL quantity perceived under FL conditions. Recent work has shown that Ca^{2+} and H_2O_2 rapidly accumulate in chloroplasts of *Chlamydomonas* in response to HL (Pivato et al., 2023). It is tempting to speculate that HL induced formation of H_2O_2 does not only directly contribute to photo-oxidative damage in chloroplasts, but also has an important role in triggering Ca^{2+}-dependent signaling pathways required for HL acclimation (Petroutsos et al., 2011). Hence, Ca^{2+}-dependent signaling might be involved in the regulation of PsbS synthesis, as well. Strikingly, an increase of Ca^{2+} in the chloroplast stroma has earlier been reported also in response to light dark transition (Sai & Johnson, 2002). Therefore, Ca^{2+}-dependent signaling might also contribute to the suppression of PsbS degradation during dark phases.

In conclusion, our work shows that growth of *Chlamydomonas* cells under discontinuous HL illumination conditions has a significant impact on photosynthetic performance and HL acclimation. In general, intermittent dark phases negatively affect cell growth and lead to a delay of HL acclimation processes, including NPQ activation and PsbS degradation. With respect to growth under natural conditions, it can be concluded that *Chlamydomonas* is most sensitive to HL stress under short-day conditions or, more precisely, under long-night conditions. Hence, dark phases are not beneficial for *Chlamydomonas*. However, FL conditions during the day period can be expected to have no negative impact on both growth and HL acclimation of the cells. For biotechnological applications, *Chlamydomonas* should thus be cultured under either constant light or without dark phases when FL conditions are applied.

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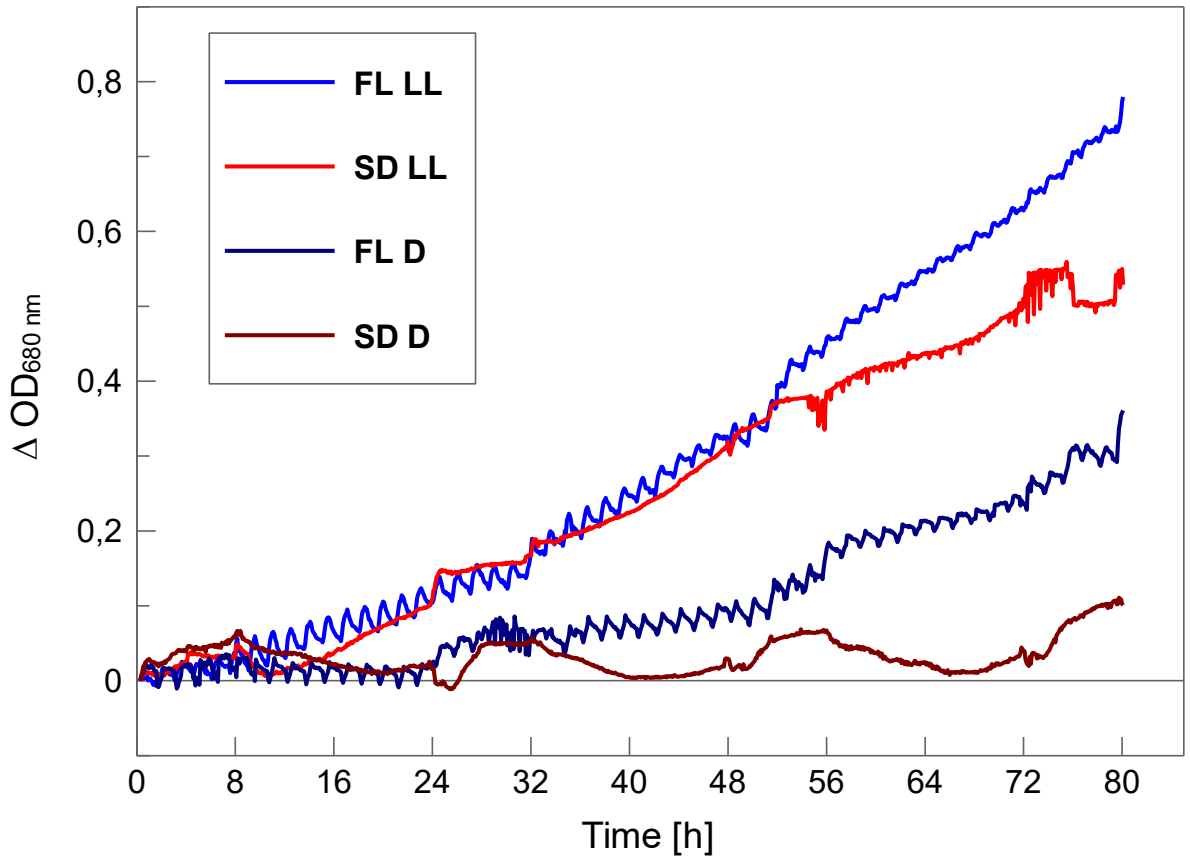

Figure S1. Growth curves.

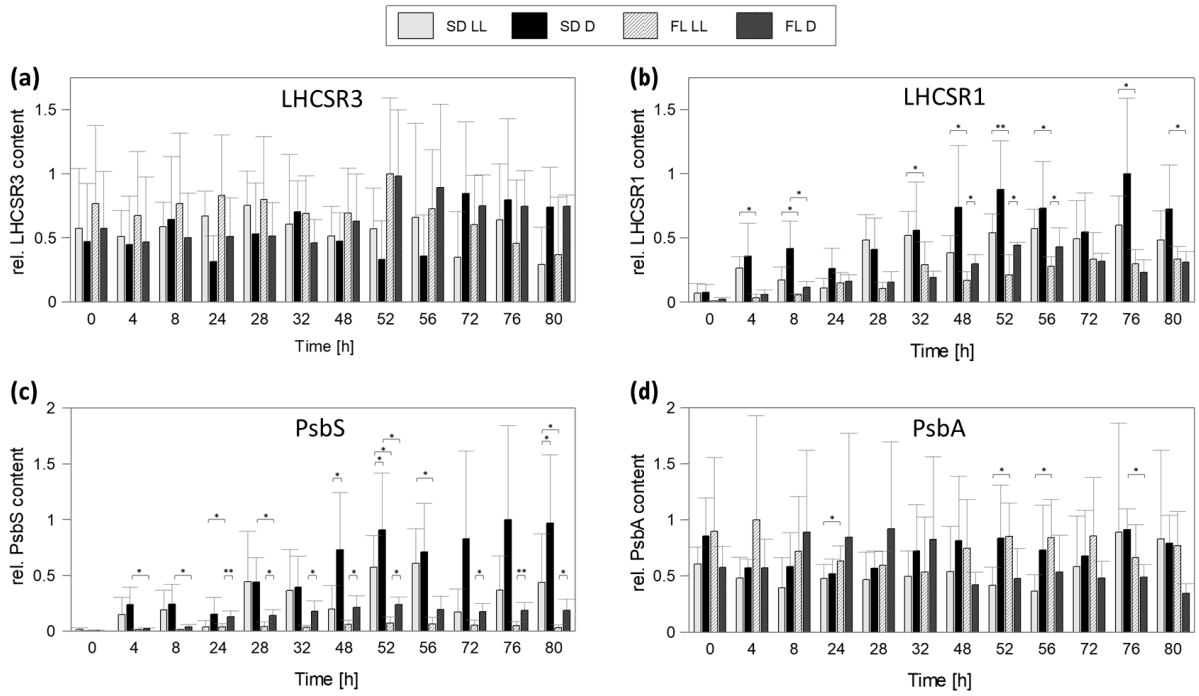

Figure S2. Statistical analysis of the protein data.

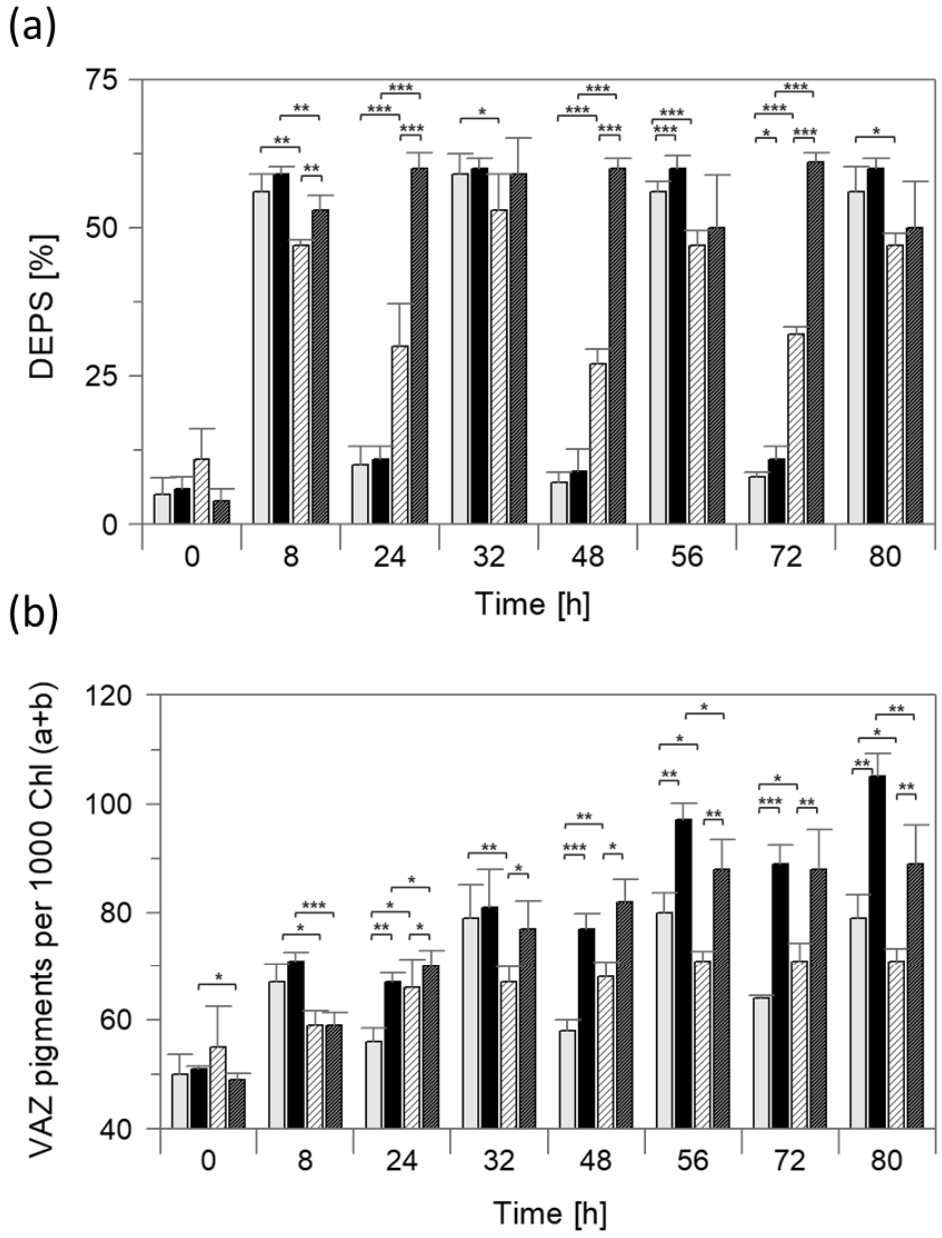

Figure S3. Statistical analysis of xanthophyll cycle pigment data.

7. MANUSCRIPT 2

Combined high light and salt stress enhances accumulation of PsbS and zeaxanthin in *Chlamydomonas reinhardtii*

Fritz Hemker, Fabian Zielasek and Peter Jahns*

Photosynthesis and Stress Physiology of Plants, Heinrich-Heine-University Düsseldorf, 40225 Düsseldorf, Germany

*Corresponding author: E-mail, pjahns@hhu.de; Fax, +49-211-8113706

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Abstract

The performance and acclimation strategies of *Chlamydomonas reinhardtii* under stress conditions is typically studied in response to single stress factors. Under natural conditions, however, organisms rarely face only one stressor at a time. Here, we investigated the impact of combined salt and high light stress on the photoprotective response of *C. reinhardtii*. Compared to the single stress factors, the combination of both stressors decreased the photosynthetic performance, while activation of energy dissipation remained unaffected. However, PsbS protein was strongly accumulated and the conversion of violaxanthin to zeaxanthin was enhanced. These results support an important photoprotective function of PsbS and zeaxanthin independently of energy dissipation under combined salt and high light stress.

1 INTRODUCTION

Photosynthetic organisms are frequently subject to a wide range of abiotic stressors, which are often interconnected and affect plants or algae in combination. Combined high light (HL) and salinity stress are typical for aquatic organisms like the green alga *Chlamydomonas reinhardtii*, e.g. in shallow waters upon increasing evaporation during sunny days. Such unfavorable environmental conditions limit photosynthesis and promote photo-oxidative stress, due to the formation of reactive oxygen species (ROS) which have a high damaging potential for all cell components (Moller et al., 2007). Therefore, photosynthetic organisms have developed several photoprotective strategies to prevent photo-oxidative damage (Li et al 2009). HL-induced acclimation responses have been widely studied in Chlamydomonas. One of the most powerful photoprotective mechanisms is non-photochemical quenching (NPQ) of excess excitation energy (Horton et al., 1996). The dominating and most flexible NPQ process is the pH-dependent qE mechanism, which is rapidly (within 1-2 min) activated and deactivated in response to changes of the thylakoid lumen pH (Nilkens et al., 2010). In Chlamydomonas, qE activation depends on LHCSR proteins which act as sensor of the lumen pH and essentially contribute to qE (Peers et al., 2009; Girolomoni et al., 2019). Both LHCSR proteins, LHCSR3 and LHCSR1, are supposed to induce energy quenching in both photosystems (Kosuge et al., 2018) with LHCSR3 being generally more efficient than LHCSR1 (Girolomoni et al., 2019; Perozeni et al., 2020). LHCSR proteins are not constitutively expressed in Chlamydomonas under continuous low light (LL), but accumulate within a few hours after onset of HL exposure (Allorent et al., 2016; Correa-Galvis et al., 2016).

In vascular plants, the PsbS protein fulfills the key role as pH sensor for qE activation (Li et al., 2000). PsbS is present in Chlamydomonas as well but has a different, yet unknown function. In Chlamydomonas, PsbS accumulates with the onset of HL but is actively degraded after only 8 to 16 h of HL (Correa-Galvis et al., 2016; Tibiletti et al., 2016; Redekop et al., 2020). These PsbS dynamics led to the assumption that PsbS might be involved in the establishment of LHCSR-mediated NPQ (Correa-Galvis et al., 2016). PsbS accumulation is further responsive to other abiotic stresses such as nitrogen starvation (Miller et al., 2010) or UV treatment (Allorent et al., 2016). Therefore, an entirely different role of PsbS in photoprotection than in vascular plants is feasible for Chlamydomonas.

Salinity stress has not been studied extensively in Chlamydomonas, so far. Chlamydomonas acclimates to salt stress similar as other unicellular organisms by decreasing cell volume and modifying lipid composition (Bazzani et al., 2021). Consequently, osmotic stress has been used to enhance the production of lipids, a valuable product in the biotechnical application of green algae (Meijer et al., 2017). Osmotic stress triggers the formation of palmelloids (Khona et al., 2016), inhibits nitrate and sulfate uptake (Shetty et al., 2019), and activates ion transporters and pumps (Vega et al., 2006). Moreover, salt stress induces formation of ROS leading to upregulation of antioxidant enzymes (Zuo et al., 2014; Vavilala et al., 2015). Aside from ROS formation, photosynthetic electron transport is affected

by salt stress at the level of plastocyanin-mediated electron transfer to PSI (Cruz et al., 2001) and the efficiency of both photosystems is reduced in response to salt stress (Neelam & Subramanyam, 2013). Here, we studied the impact of combined stress of HL (600 µmol photons m^{-2} s^{-1}) and salinity (25, 50 or 100 mM NaCl) on the photoprotective response of Chlamydomonas. Our data indicate that specifically the PsbS protein and the xanthophyll zeaxanthin accumulate under those conditions, supporting a critical role of PsbS and zeaxanthin under combined HL and salt stress.

2 MATERIAL AND METHODS

2.1 Strains and growth conditions

The wild-type strain cc124 was used for all salt measurements. The cultures were grown in TAP medium for one week and subsequently transferred to HSM media for optimal growth in photoautotrophic conditions at 30 µmol photons m^{-2} s^{-1}. The culture was split in glass tubes specific for Multicultivator MC 1000-OD Mix (Photon Systems Instruments, Czech Republic) and grown until reaching an optical density (OD) at 720 nm of 0.6. The first sample (0h) was collected and, afterwards, salt solution was added to 25, 50 or 100 mM of NaCl in addition to the salt in the HSM medium which mirrors the perfect growth conditions. In 0 mM samples, HSM medium was added to the same volume. The cultures were illuminated with either LL (30 µmol photons m^{-2} s^{-1}) or HL (600 µmol photons m^{-2} s^{-1} + 200 µW/cm^2 UV-B light). UV-B was added to ensure that expression of UV-B responsive genes is not limited by a possibly low portion of UV-B provided by the HL source. Samples were collected after 0, 3, 6, 24 and 48 h. Growth was measured at ODs of 680 nm and 720 nm.

2.2 Chlorophyll fluorescence analyses

Chlorophyll fluorescence measurements were performed with Fiber-PAM (Walz, Effeltrich, Germany). Following dark incubation under shaking at 100 rpm for 25 min, the samples were transferred to a glass fiber filter (Pall, Dreieich, Germany). For measurements, the samples were exposed for 1 min to far-red light (200 µmol photons m^{-2} s^{-1}) followed by 10 min to red actinic light (1,300 µmol photons m^{-2} s^{-1}) and finally 5 min to darkness. Every minute, saturation pulses of 10,000 µmol photons m^{-2} s^{-1} were applied. NPQ was calculated as (Fm/Fm') - 1 and qE was calculated as the difference of NPQ value at the end of the actinic light and the NPQ value after 1 min of dark relaxation.

2.3 Protein analyses

Protein samples were spun down and resolved in 150 µL lysis and protein extraction buffer (1.6% SDS, 1 M urea, 50 mM Tris/HCl pH 7.6). For PsbS detection, 30 µg of total protein were loaded while for the LHCSR proteins, 5 µg were loaded. Protein concentration was determined using the DC protein assay (Bio-Rad, Feldkirchen, Germany). SDS-PAGE was performed according to (Laemmli, 1970; Kyhse-Andersen, 1984). The antibodies in use were PsbS (Pineda antibody service, Berlin, Germany), LHCSR3 (AS14 2766) and LHCSR1 (AS14 2819) (both from Agrisera, Vännas, Sweden). The detection was performed in ChemiDoc (Bio-Rad, Feldkirchen, Germany) using Milliipore Immobilon Western HRP substrate (Merck, Darmstadt. Germany)

2.4 Pigment analyses

HPLC samples were spun down, washed three times with ddH_2O and resolved in 1 mL acetone (100%). The pigment extract was spun down again, the supernatant was filtered (0.2 µm pore size) and used for reversed phase HPLC (Färber et al., 1997).

2.5 Statistical analyses

Statistical analyses were performed using student's t-test. Significant differences are indicated by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3. RESULTS

3.1 General fitness

Cell growth and the Fv/Fm ratio of chlorophyll fluorescence were determined to address the general fitness of the cells under single stress of salt and combined stress of HL and salt. In LL acclimated cells, addition of 50 mM and 100 mM NaCl induced an arrest of cell growth and led to reduction of the cell density (Figure 1A). Under HL conditions, growth was generally higher, but cell growth was markedly reduced with increasing salinity stress (25 mM and 50 mM NaCl), as well (Figure 1A). Further increase of the salt concentration to 200 mM NaCl under LL conditions and to 100 mM NaCl under HL conditions led to a rapid bleaching of the cells and hence irreversible damage of the cells (not shown). The negative impact of salt stress is also visible at the level of the PSII chlorophyll fluorescence parameter Fv/Fm (Figure 1B). Under LL conditions, high salt concentrations significantly reduced Fv/Fm, resulting in stable minimum Fv/Fm values of about 0.65 after 48 h of stress exposure. When salt stress was combined with HL stress, the drop of Fv/Fm was even more pronounced. However, this was predominantly related to the salt-independent reduction of Fv/Fm by HL treatment to values of about 0.65 after 48 h and a transient decrease to about 0.55 after 3 h HL. Addition of salt further reduced the Fv/Fm by about 0.1 units to values of about 0.45 after 48 h upon addition of 50 mM NaCl. The impact of salt stress on Fv/Fm is thus similar in combination with HL compared to LL. It should be noted, however, that the salt concentrations applied under HL conditions were a factor 2 lower than under LL.

3.2 Energy dissipation

The activation of energy dissipation in response to HL and salt stress is shown in Figure 2. As expected, salt stress alone did not trigger any NPQ response within 48 h under LL conditions (Figure 2A), because qE activation is known to require HL acclimation. In response to HL, however, the typical establishment of a high qE capacity to values of about 1 after 24 h of HL exposure was detectable. Addition of 25 mM salt had no impact on the induction of qE capacity, but in presence of 50 mM of salt, the induction of the maximal qE capacity seemed to be delayed, as can be derived from the significantly lower qE after 24 h of HL exposure (Figure 2B), either due to a lower NPQ capacity or due to lower electron transfer under salt stress. After 48 h, however, same maximum qE values as in absence of salt were found also in presence of 50 mM NaCl. Hence, salt stress has no or only very limited impact on qE activation.

3.3 Accumulation of photoprotective proteins

Like activation of qE, the expression of photoprotective proteins (LHCSR3, LHCSR1 and PsbS) is known to require HL as well. Consequently, none of these proteins was detectable under LL, neither in absence nor in presence of salt (Figure 3). In response to HL, however, all proteins were rapidly expressed within 3 h. LHCSR3, the main driver of qE, accumulated gradually in absence and presence of salt stress. The addition of salt did not lead to pronounced differences, but in tendency the LHCSR3 amount was reduced under combined HL and salt stress conditions (Figure 3). The LHCSR1 content

reached maximal amounts within 6 h of HL and the addition of salt rather led to slightly, though not significantly increased LHCSR1 accumulation (Figure 3). Only for PsbS, significant differences in protein accumulation were detectable (Figure 3B). In absence of salt, PsbS showed the well-known accumulation pattern in response to HL with maximum levels after 3 to 6 h and nearly complete degradation within 24 h. Upon addition of salt, however, PsbS accumulated to about 3-fold higher amounts within 6 h, and the subsequent degradation of PsbS was delayed (Figure 3B). Obviously, specifically the accumulation of PsbS was increased in response to combined HL and salt stress.

3.4. Xanthophyll cycle activity

The amount and the de-epoxidation state (DEPS) of the xanthophyll cycle pigments (VAZ pool) are further indicators of light utilization and photo-oxidative stress, respectively. Under LL conditions, the DEPS remained below 5% in absence of salt, but was significantly increased to values of up to 20% in response to salt stress (Figure 4A), indicating that violaxanthin to zeaxanthin conversion is stimulated by salt stress. As expected, a strong increase of the DEPS to 50-60% was induced within 3 h of HL exposure in absence and presence of salt, but the DEPS remained at higher values in salt treated samples compared to cells treated by HL only (Figure 4A). The VAZ pool size remained nearly unchanged under LL conditions at values of about 60 VAZ / Chl (a+b), independently from the absence or presence of salt (Figure 4B). HL exposure induced an increase of the VAZ pool size to 90 VAZ / Chl (a+b) in absence of salt, but to significantly higher values of about 110 VAZ / Chl (a+b) in presence of salt after 48 h (Figure. 4B).

DISCUSSION

Our results provide evidence that salinity stress impairs cell growth, independently of the growth light conditions (Figure 1A). The suppression of growth agrees with former work which related the impairment of growth by salinity stress to the formation of ROS (Zuo et al., 2014; Vavilala et al., 2015). Photosynthetic electron transport seemed to be only mildly affected under our conditions, as can be derived from the only moderately reduced efficiency of PSII (Figure 1B). Increased ROS formation will result in oxidative stress and can be expected to induce typical oxidative stress responses and acclimation processes. Indeed, an increase of the antioxidative defense system has been reported in response to salt stress (Zuo et al., 2014; Vavilala et al., 2015). Here, we show that typical photoprotective responses are differentially affected by salt stress. The HL induced activation of energy dissipation including the expression of LHCSR proteins was not altered in response to additional salt stress (Figures 2 and 3), underlining that the general HL acclimation response is unaffected. The same conclusion derives from the partial recovery of Fv/Fm after 24 h of HL exposure when compared to the initial drop after 3 h HL (Figure 1B). However, PsbS accumulation (Figure 3) and the DEPS of the xanthophyll cycle (Figure 4A) pigments showed clear differences from the known HL acclimation response. Strikingly, PsbS accumulated in response to combined HL and salt stress to up to 3-fold higher amounts compared to single HL stress (Figure 3), supporting an important role of PsbS independently from NPQ, as has been proposed recently (Redekop et al., 2020). Moreover, salt stress induced an increase of the DEPS under both light conditions (Figure 4A) in combination with an increase of the VAZ pool size, which was more pronounced and significant under HL conditions but as trend also detectable under LL conditions (Figure 4B). Consequently, higher amounts of zeaxanthin accumulate in the thylakoid membrane in response to salt stress. Zeaxanthin has been shown to modify the thylakoid membrane stability (Havaux, 1998) and to have an important photoprotective function unrelated to NPQ, likely as antioxidant in the lipid phase of the membrane (Havaux & Niyogi, 1999). Since salt stress is also known to affect the lipid composition in Chlamydomonas (Bazzani et al., 2021), an increase of the zeaxanthin amount in response to salt stress might thus contribute to the stabilization of the membrane. Similarly, the strongly increased PsbS amount in response to salt stress might be related to altered membrane properties. The activation of qE in vascular plants involves conformational changes of the organization of PSII antenna proteins which are likely controlled by PsbS (Horton et al., 1996). Based on its high hydrophobicity and its compact structure (Fan et al., 2015), PsbS is ideally suited to interact with different membrane proteins and thus to control the organization of other proteins in the membrane. The pH-controlled switch between the monomeric and dimeric state of PsbS (Bergantino et al., 2003) further promotes the flexible reorganization of thylakoid membrane proteins by PsbS in response to lumen acidification. Salt stress-induced changes in the composition can be expected to impact the organization of protein complexes in the thylakoid membrane in Chlamydomonas. Increased accumulation of PsbS protein during combined HL and salt stress can thus be understood as a critical function of PsbS in the (re-)organization of thylakoid membrane proteins during HL acclimation.

AUTHOR CONTRIBUTIONS

Fritz Hemker and Peter Jahns designed the research; Fritz Hemker and Fabian Zielasek performed the experiments; Fritz Hemker and Fabian Zielasek analyzed data; Fritz Hemker and Peter Jahns wrote the original draft; Fritz Hemker, Fabian Zielasek and Peter Jahns reviewed and edited the manuscript.

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DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as all new created data is already contained within this article.

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Supporting information
Additional supporting information can be found online in the Supporting Information section at the end of this article
Figure S1: Linearity of Western Blot signal intensities

FIGURES

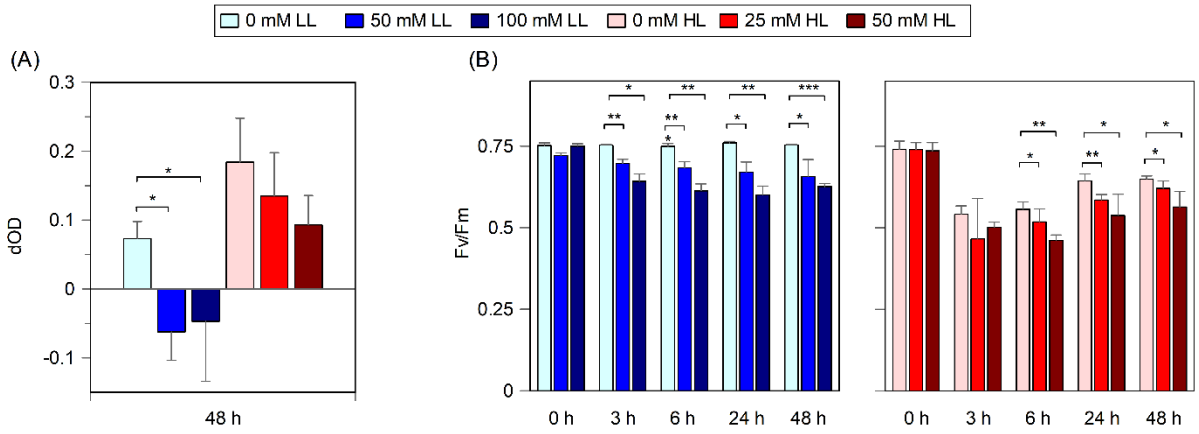

FIGURE 1 Growth and photosynthetic parameters. (A) Cell growth was derived from the optical density at 720 nm. The difference of the OD before and after 48 h of treatment with indicated salt concentrations is shown (dOD). Data represent mean values + SD of 3 biological replicates (n = 3). (B) Fv/Fm was determined by chlorophyll fluorescence analyses of samples taken at indicated time, and after 25 min of dark adaptation. Mean values + SD of 3 biological replicates (n = 3) are shown for low light (LL) grown cells and of 4 biological replicates for high light (HL) grown cells. Cells were grown at indicated concentrations of NaCl under either LL (30 µmol photons m^{-2} s^{-1}) or HL (600 µmol photons m^{-2} s^{-1} + 200 µW/cm^2 UV-B light). Brackets with asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$,) between salt treated cells and the corresponding control (0 mM NaCl).

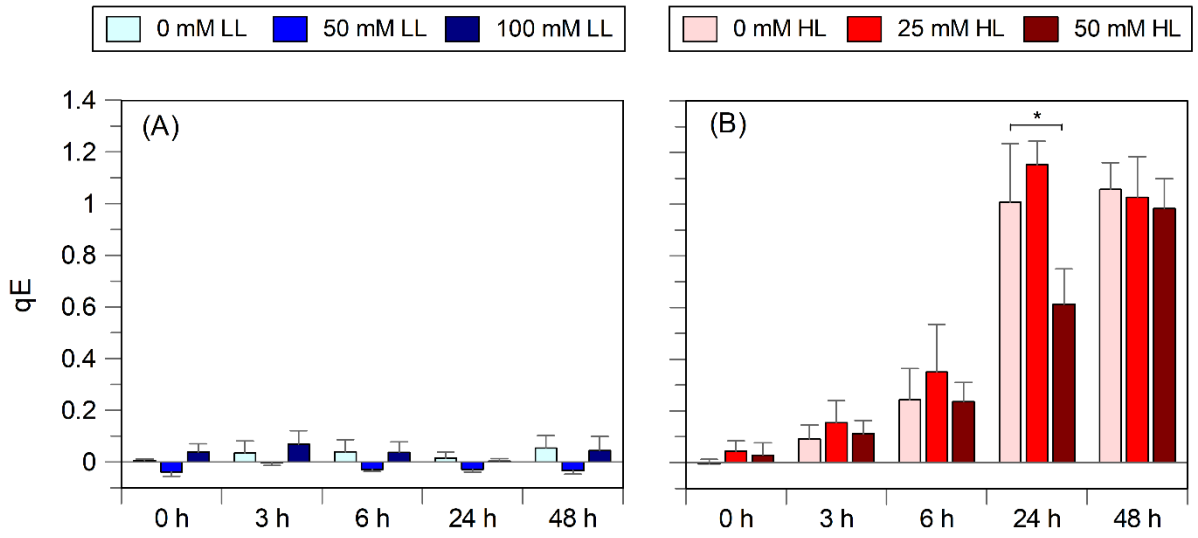

FIGURE 2 qE capacity. NPQ induction (at 1,800 µmol photons m^{-2} s^{-1}) and relaxation (in the dark) was derived from chlorophyll fluorescence measurements. The qE capacity was defined as the difference of the maximum NPQ at the end of the actinic light and the NPQ value after 1 min of dark relaxation. Mean values + SD of 3 biological replicates (n = 3) are shown for (A) low light (LL = 30 µmol photons m^{-2} s^{-1}) grown cells and of 4 biological replicates for (B) high light (HL = 600 µmol photons m^{-2} s^{-1} + 200 µW/cm^2 UV-B light) grown cells. Cells were grown at indicated concentrations of NaCl under either LL or HL. The bracket with an asterisks indicates significant difference (* $p < 0.05$) between salt treated cells (50 mM HL) and the corresponding control (0 mM salt).

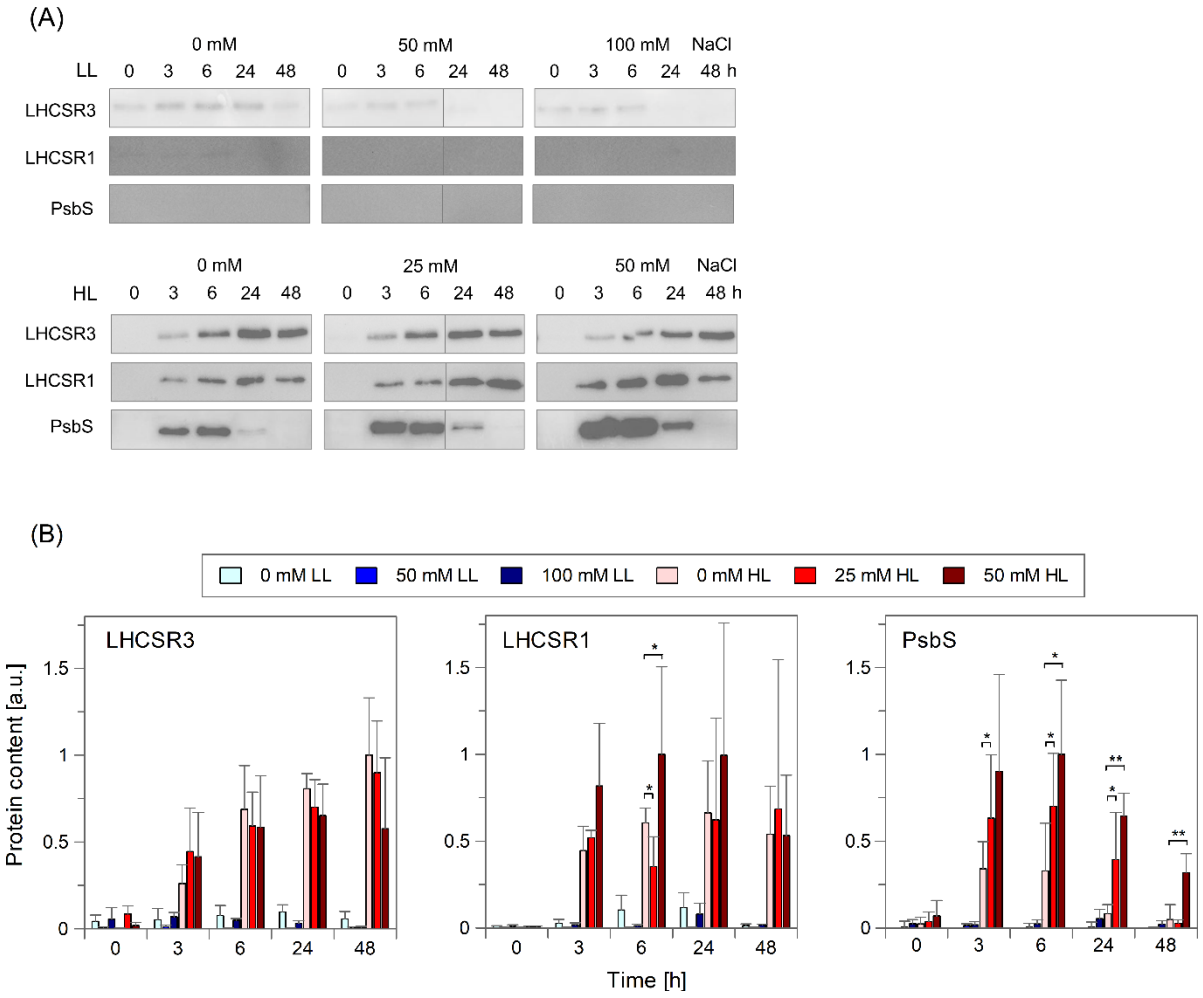

Figure 3 LHCSR and PsbS content. The relative amount of the proteins LHCSR3, LHCSR1 and PsbS was derived from Western Blot analyses. (A) Representative original Western blot. (B) Relative protein amount as quantified by densitometric scans of the corresponding blots. Cells were grown at indicated concentrations of NaCl under either LL (30 µmol photons m^{-2} s^{-1}) or HL (600 µmol photons m^{-2} s^{-1} + 200 µW/cm^2 UV-B light). Data represent mean values +SD of 4 biological replicates (n = 4). Asterisks indicate significant differences (* $p < 0.5$, ** $p < 0.01$) between salt treated cells and the corresponding control (0 mM salt).

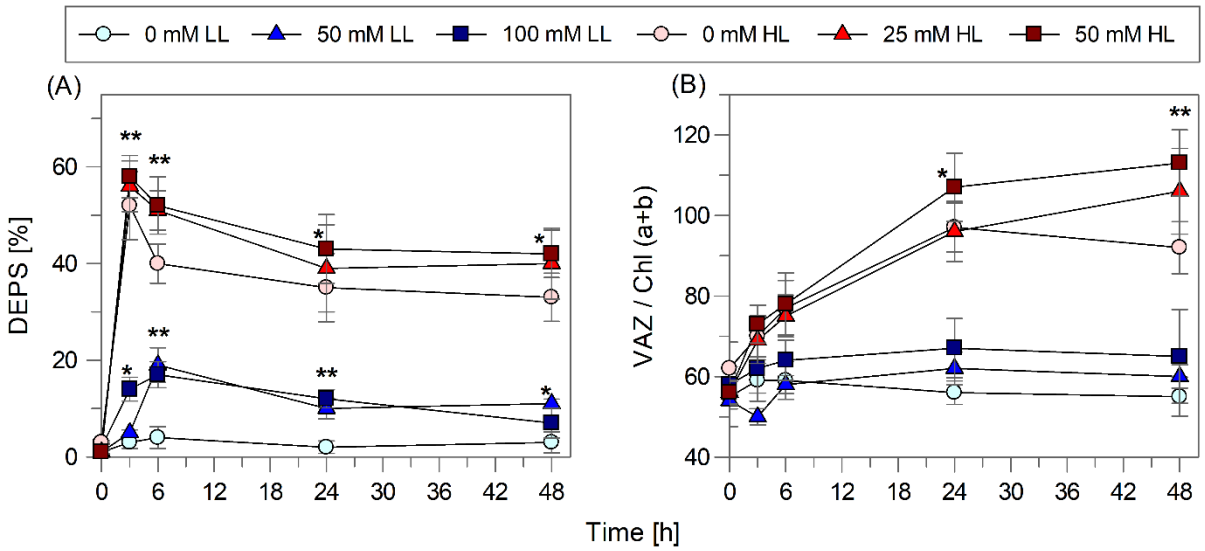

Figure 4 Xanthophyll cycle pigments. The pigment composition of the cells was determined by HPLC analysis. (A) The de-epoxidation state (DEPS) of the xanthophyll cycle pigments. The DEPS of violaxanthin (Vx), antheraxanthin (Ax) and zeaxanthin (Zx) is defined as (0.5 Ax + Zx) / (Vx + Ax + Zx)*100. (B) The sum of the xanthophyll cycle pigments, VAZ / Chl (a+b). The amount of VAZ pigments (= VAZ pool size) is given in relation per 1000 Chl (a+b). Mean values of 3-4 biological replicates (n = 3-4) ± SD are shown. Cells were grown at indicated concentrations of NaCl under either LL (30 µmol photons m^{-2} s^{-1}) or HL (600 µmol photons m^{-2} s^{-1} + 200 µW/cm^2 UV-B light). Asterisks indicate significant differences ($p < 0.05$) between salt treated cells and the corresponding control (0 mM salt). * = significant difference between control and a single salt concentration. ** = significant difference between control (0 mM NaCl) and both salt concentrations.

8. MANUSCRIPT 3

***Chlamydomonas reinhardtii* PsbS has no immediate impact on fitness or non-photochemical quenching under high light**

Fritz Hemker[a], Petra Redekop[b,c]*, Neda Fakhimi[b], Timothoy Nicodemus[c], Weichao Huang[b], Christoph Benning[d], Arthur R. Grossman[b], Peter Jahns[a]*

[a]Photosynthesis and Stress Physiology of Plants, Heinrich-Heine-University Düsseldorf, 40225 Düsseldorf, Germany

[b]Department of Plant Science, Carnegie Institution of Science, Stanford, CA 94305, United States

[c]Plant Physiology, Freie Universität Berlin, 14195 Berlin, Germany

[d]Plant Biology Laboratories, Michigan State University, USA

*Correspond to either author: petra.redekop@fu-berlin.de or pjahns@hhu.de

Status: submission in preparation

ABSTRACT

Non-photochemical quenching (NPQ) of excess excitation energy is a powerful strategy of photosynthetic organisms to reduce photo-oxidative damage under high light stress conditions. In vascular plants, the PsbS protein is the key regulator of NPQ related to its function as sensor of the thylakoid lumen pH. In the green algae *Chlamydomonas reinhardtii*, this regulatory function is fulfilled by LHCSR proteins, while the role of the PsbS remains elusive. We generated PsbS knockout lines and studied their performance under constant and fluctuating high light conditions. Lack of PsbS did not result in any noticeable phenotype under the chosen experimental conditions. Neither the activation of NPQ including the synthesis of LHCSR proteins, nor the general fitness of the cells were affected in absence of PsbS. Only the de-epoxidation state of the xanthophyll cycle pigments was found to be reduced in PsbS knockout lines compared to wild-type cells. In agreement with earlier results obtained with PsbS knock-down lines, our work excludes an essential role of PsbS in NPQ, but supports a function of PsbS as regulator of the thylakoid membrane organization.

INTRODUCTION

Under natural conditions, photosynthetic organisms have to cope with a variety of abiotic stress factors. Since all stressors typically limit the photosynthetic capacity of plants and algae, most stress conditions go along with photo-oxidative stress. Hence, understanding of the key parameters that are involved in the acclimation of plants and algae to high light (HL), is highly important. One of the most powerful defense strategies against photo-oxidative damage, which is active in most photosynthetic organisms, is the non-photochemical quenching (NPQ) of excitation energy. Activation of NPQ leads to the dissipation of excess excitation as heat and by that reduces the light-induced damage of key cellular elements (Demmig-Adams et al., 1996; Horton et al., 1996). The dominating and fastest component of NPQ is the pH-regulated qE mechanism, which is activated by acidification of the thylakoid lumen (Tokutsu and Minagawa, 2013). In the green alga *Chlamydomonas reinhardtii,* the proton gradient, or more precisely the low lumen pH, activates two photoprotective proteins, LHCSR3 and LHCSR1, which in turn activate qE-quenching in the antenna of photosystem II (PSII) (Bonente et al., 2011; Kosuge et al., 2018; Peers et al., 2009). The major portion of qE and hence of NPQ is controlled by LHCSR3, which is the main quencher of excess light in PSII (Girolomoni et al., 2019). LHCSR1 is supposed to contribute to NPQ mainly at PSI (Kosuge et al., 2018; Perozeni et al., 2020). Notably, the LHCSR proteins are ancient proteins that were lost during evolution of higher plants (Alboresi et al. 2010). Vascular plants rely on PsbS as pH sensor and key regulator of qE (Bergantino et al., 2003; X. P. Li et al., 2000). Together with zeaxanthin (Zx) and lutein, PsbS is mandatory for qE activation in plants (Bonente et al., 2008; Niyogi et al., 1997). In contrast to LHCSR proteins in Chlamydomonas, however, PsbS in land plants is not a direct quencher of energy, but rather activates qE in PSII antenna proteins through interaction with LHCII subunits (Correa-Galvis, Poschmann, et al., 2016; Sacharz et al., 2017). It has been long assumed that *C. reinhardtii* does not possess PsbS, but upon publication of the *C. reinhardtii* genome, two nearly identical genetic copies of PsbS were identified (Anwaruzzaman et al., 2004). In *C. reinhardtii*, PsbS accumulates only transiently during HL exposure and is degraded after 8-16 h of HL (Correa-Galvis, Redekop, et al., 2016; Tibiletti et al., 2016). Further regulators of PsbS accumulation are CO_2 availability (Correa-Galvis, Redekop, et al., 2016) and UV-B light (Allorent et al. 2016) (Redekop et al., 2022). CrPsbS is unable to compensate the loss of LHCSR proteins at the level of qE and CrPsbS was suggested to be involved in the establishment of qE during early stages of HL acclimation (Correa-Galvis, Redekop, et al., 2016), but PsbS over-expressing lines did not show increased NPQ capacity (Redekop et al., 2020). Up to now, the precise function of PsbS in *C. reinhardtii* is not understood, but a direct involvement in qE appears unlikely. Other photosynthetic organisms possessing both PsbS and LHCSR proteins use both for photoprotection via qE, such as the moss *Physcomitrella patens* (Gerotto et al., 2011) or the multicellular green alga

Ulva prolifera (Zheng et al., 2019). Aside from the involvement in qE, it has been speculated that PsbS might contribute to membrane fluidity in *Arabidopsis*, in context with the formation of a quenching state of LHCII proteins (Goral et al., 2012), but is unknown to what extent this may apply to green algae as well. Deciphering the precise role of PsbS in *C. reinhardtii* would be of high interest not only for understanding the evolution of photoprotection, but also for identifing possible key elements for optimization of photosynthetic efficiency in order to enhance crop yields, especially as green algae move into the focus of agriculture yet again.

In our study, we analyzed the photosynthetic performance and photoprotection in PsbS knock-out mutants of *C. reinhardtii*, which were generated by CRISPR/Cas9 mediated genome editing, during acclimation to constant and fluctuating high light. Our results do not provide any evidence for a role of CrPsbS in NPQ, but rather support a function of CrPsbS in the regulation of thylakoid membrane properties.

MATERIAL & METHODS

Growth conditions

PsbS knock-out mutants were generated by CRISPR/Cas9 in the background of the wild type cc125. Both PsbS genes were knocked out in the mutant lines *29-2* and *320*. All cells were cultivated in TAP medium and transferred to TP medium four days prior to the start of the experiments. The cells were illuminated with cold white light at 30 µmol photons m^{-2} s^{-1} in Multi-Cultivator MC 1000-OD-MULTI (Photon Systems Instruments, Czech Republic) under constant bubbling with sterilized air. For HL treatment, the cultures were illuminated with 600 µmol photons m^{-2} s^{-1} upon addition of 200 µW/cm^2 UV-B light to ensure maximal PsbS expression. For constant light treatment, the first sample was taken in low light (LL, 0 h), just before the onset of HL illumination. All following samples were taken at the indicated time of HL exposure. For fluctuating light, the first sample was taken from LL as well, before starting a light rhythm of 15 min HL and 15 min darkness, and all other samples were analyzed at indicated time at the end of the corresponding HL phase. For each sample, 11 mL were taken from the respective culture with 4 mL being used for chlorophyll fluorescence analysis, 6 mL for Western Blot analysis and 1 mL for pigment analysis via HPLC. Growth was monitored as optical density (OD) at 720 nm.

Chlorophyll fluorescence analysis

Chlorophyll fluorescence measurements were performed with Fiber-PAM (Walz, Effeltrich, Germany). Before each measurement, samples were dark adapted for 25 min under continuous shaking at 100 rpm. The samples were placed onto a glass fiber filter (Pall, Dreieich, Germany) which was fixed in a leaf cuvette in the Fiber-PAM. The sample was exposed to 1 min of far-red light (200 µmol photons m^{-2} s^{-1}) followed by 10 min of red actinic light (1,300 µmol photons m^{-2} s^{-1}) and then 5 min darkness. Saturating light pulses (200 ms duration) of 10,000 µmol photons m^{-2} s^{-1}) were applied every minute. NPQ was calculated as (Fm/Fm') - 1, with Fm and Fm' being the maximum fluorescence in the dark- and light-adapted state, respectively (Krause & Jahns, 2003). qE was calculated by subtracting NPQ at the last measurement in the light minus the first measurement in the dark.

Protein analysis

6 mL culture was spun down and cells were resuspended in lysis and protein extraction buffer (1.6% SDS, 1 M urea, 50 mM Tris/HCl pH 7.6). Cell debris and insoluble particles were spun down and the protein concentration of the supernatant was determined using the DC protein assay (Bio-Rad, Feldkirchen, Germany). SDS-PAGE and immunoblot analyses were performed as described in (Kyhse-Andersen, 1984; Laemmli, 1970). For PsbS, 30 µg of total

protein and for LHCSR proteins, 5 µg of total protein were loaded on each lane. PsbS antibody was produced by Pineda antibody service (Berlin, Germany) and LHCSR3 (AS14 2766) and LHCSR1 (AS14 2819) antibodies were obtained from Agrisera (Vännas, Sweden). Detection was performed with Millipore Immobilon Western HRP substrate (Merck, Darmstadt, Germany) in ChemiDoc (Bio-Rad, Feldkirchen, Germany). Quantification of proteins was performed with ImageJ software (Schneider et al., 2012).

Pigment analysis

1 mL of culture was harvested by centrifugation and prepared for pigments analysis via HPLC. Pigment samples were washed with ddH_2O two times and taken up in 1 mL acetone (100%). Cell debris was pelleted via centrifugation and the supernatant was filtered (0.2 µm pore size) and used for reverse-phase HPLC (Färber et al., 1997).

Statistical analysis

Statistical analyses were performed using student's t-test. Significant differences are indicated by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

RESULTS

Two *PSBS* knock-out mutants *29-2* and *320* and the corresponding wild type cc125 were analyzed regarding photosynthetic fitness and activation of photoprotection under constant and fluctuating high light conditions. Under constant HL conditions, cell growth (OD_{720}, Fig. 1A) and PSII quantum yield (Y(II), Fig. 1B) did not show any significant differences between the WT and the two knock-out lines. Whereas all strains displayed a similar continuous growth, HL exposure led to a severe decrease in Y(II), which did not recover till the end of the experiment. The extent of the pH-regulated NPQ component qE, was used as a measure for the activation of energy dissipation. Maximum qE values of 1.5-1.8 were reached in all lines after about 48 h of HL acclimation (Fig. 1C). No significant differences were detectable when comparing WT cells and the two mutant lines, although mutant line *320* displayed a slightly lower qE than line *29-2* and WT cc125 (Fig. 1C).

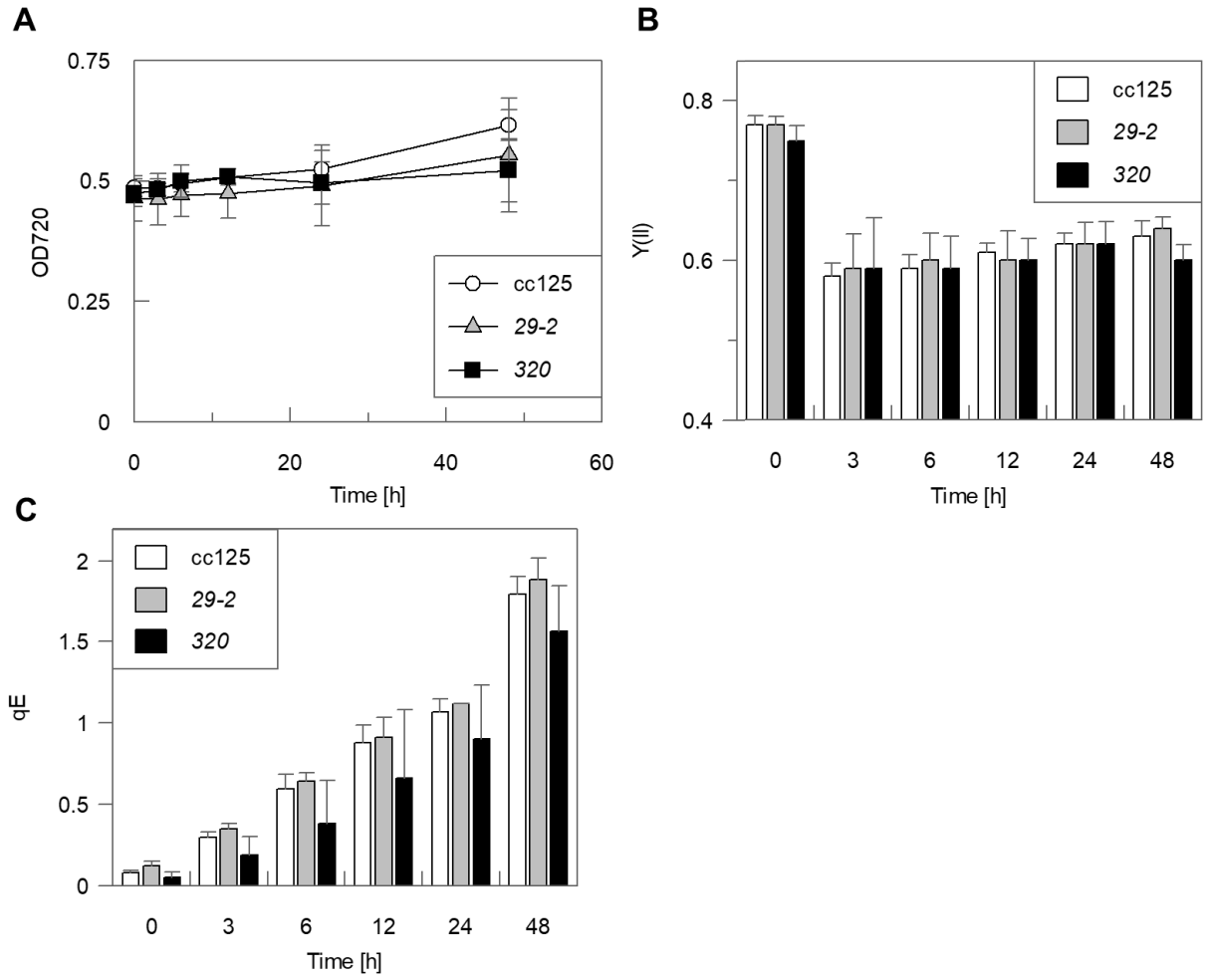

Figure 9: Fitness and stress parameters under constant HL conditions. Growth via OD720 (A), quantum yield of photosystem II (B) and qE capacity (C) are shown for WT cc125 and PsbS mutants *29-2* and *320*. Data represent mean values +/- SD of 3-4 biological replicates. Brackets with asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) and small shapes ($p < 0.05$) indicate significant differences compared to the wild type.

Applying Western Blot analyses, we further examined the impact of the loss of PsbS on the accumulation of the photoprotective LHCSR proteins (Fig. 2 and S1). Complete loss of PsbS in the two mutant lines *29-2* and *320* was verified in immunoblots with an antibody specific for Chlamydomonas PsbS. Whereas WT cells showed the typical transient accumulation of PsbS in response to constant HL, no PsbS was detectable in lines *29-2* and *320* (Fig 2A). The two LHCSR proteins accumulated straight after the onset of HL (Fig. 2B and C). The highest accumulation for LHCSR3 was monitored after 12 h of HL. Although some significant differences between the LHCSR3 content of WT and PsbS knock-out mutants have been detected, those differences did not appear to follow any clear pattern. For LHCSR1, no significant differences were detected at all. Obviously, the loss of PsbS protein does not lead to an altered accumulation of the LHCSR proteins.

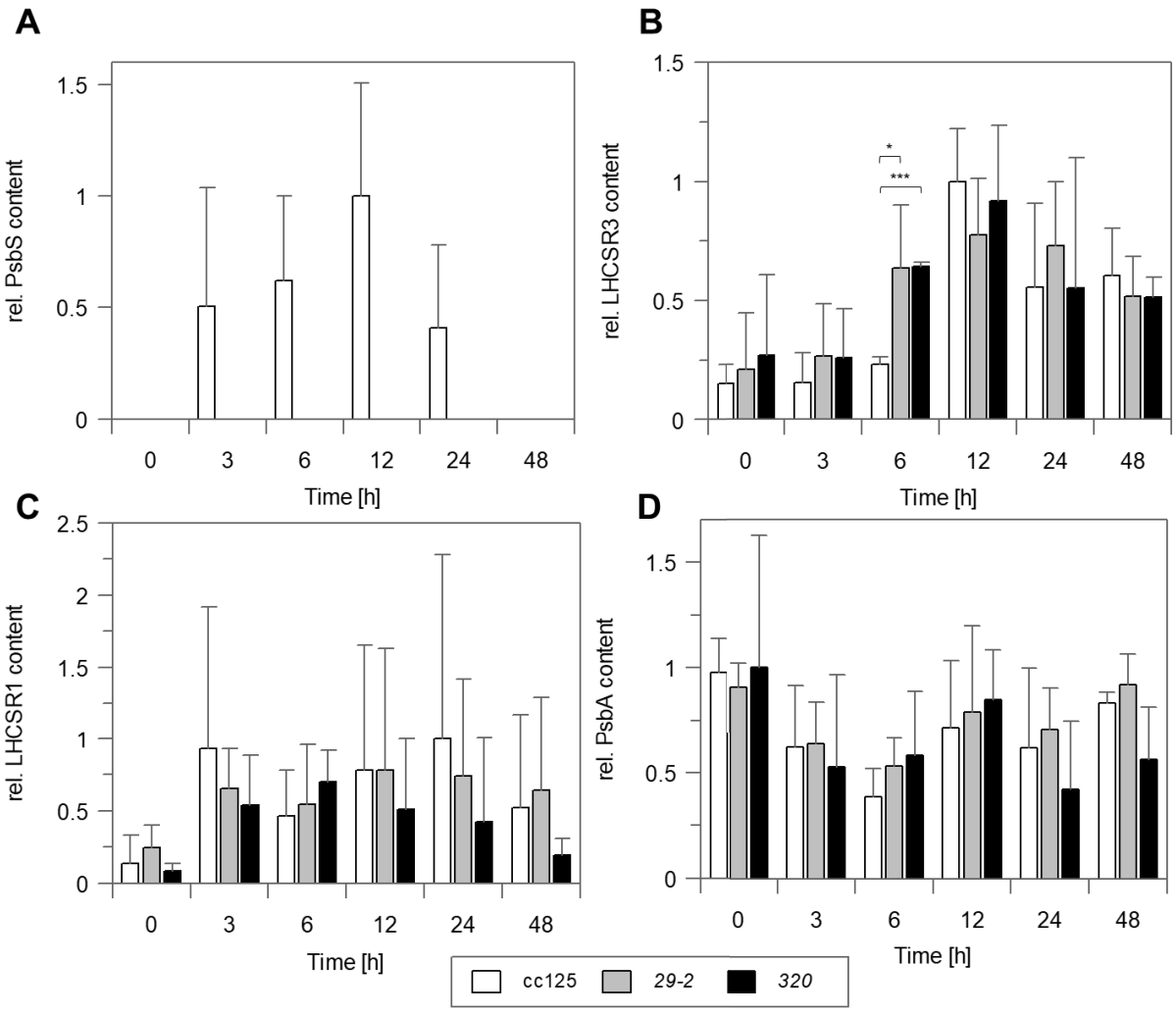

Figure 10: Protein accumulation under constant HL in WT and PsbS-deficient mutants *29-2* and *320*. The accumulation patterns of PsbS (A), LHCSR3 (B), LHCSR1 (C) and PsbA (D) are shown. No PsbS was detectable in the mutants at any point and in the WT before HL initiation and after 48 h of constant HL. Mean values + SD of 3 biological replicates are shown. Brackets with asterisks indicate significant differences (*, $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

As no significant changes were detectable in the dynamics of qE induction as well, it is unlikely that PsbS is involved in the establishment of qE. Moreover, the de-epoxidation state (DEPS) of the xanthophyll cycle pigments was analyzed to obtain information about the amount of the photoprotective xanthophyll Zx. The DEPS rose to values of about 60% in all genotypes within 3 h of HL exposure, but subsequently dropped to values of about 30% within 12 h of HL acclimation (Fig. 3A). While no differences were detectable between WT and mutant *29-2* cells, slightly lower DEPS values were determined for mutant *320* after 24h and 48 h of HL exposure. Irrespective of this slight reduction in one of the mutant lines, it can be concluded that the absence of PsbS has no impact on the xanthophyll conversion in response to constant HL. This is also mirrored by the lack of significant differences in the VAZ pool size (Fig. 3B). The analysis of the antheraxanthin (Ax) accumulation showed that the mutant *320* accumulated slightly lower levels of Ax in comparison with the wild type after more than 6h of HL exposure (Fig. 3C).

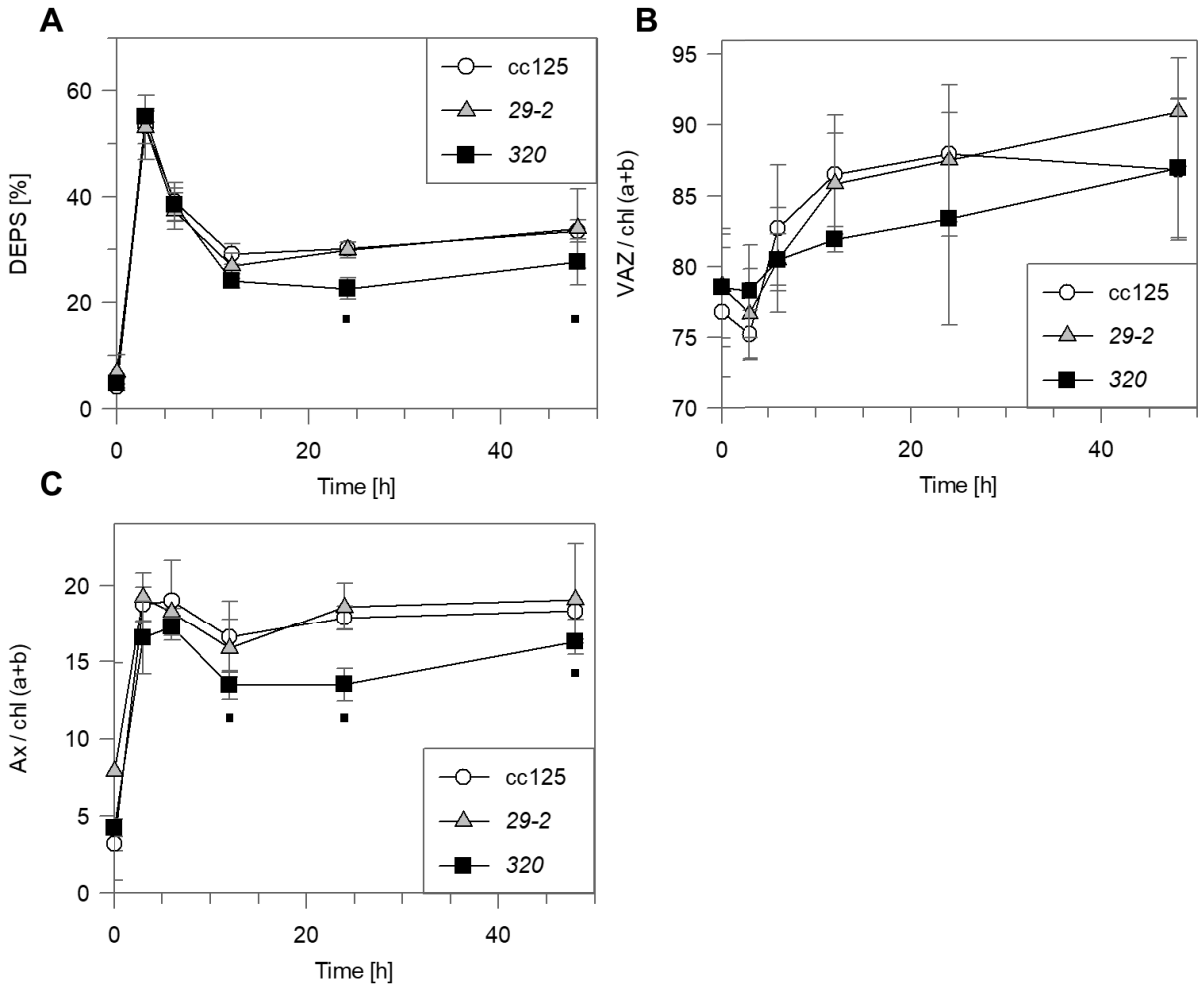

Figure 11: Pigment composition of WT and PsbS-deficient strains under constant high light conditions. The de-epoxidation state of xanthophyll pigments (A), the total VAZ pool size (B) and the amount of the xanthophyll antheraxanthin (C) are shown. Mean values +/- SD of 3-4

biological replicates are shown. Small shapes ($p < 0.05$) indicate significant differences compared to the wild type.

The transient accumulation of PsbS during HL acclimation and its mandatory light- and CO_2-dependent degradation led to the speculation about an essential photoprotective role of PsbS under fluctuating light conditions. Here, we investigated the performance of the *PSBS* knock-out mutants under rapidly fluctuating light conditions (15 min HL and 15 min dark). As under constant HL conditions, no significant differences were detectable between WT and mutant lines with respect to the quantum yield of PSII (Fig. 4A). However, the HL induced drop of Y(II) was less pronounced than under constant HL exposure. The HL induced establishment of the qE capacity under FL conditions followed similar dynamics than under constant HL without any pronounced differences between WT and mutant lines (Fig. 3B). Moreover, the maximum qE capacity after 48 h growth under FL conditions was similar as after 48 h of growth under constant HL. Comparing WT and mutant lines, the qE capacity in the mutants was similar or even slightly increased, excluding again any critical role of PsbS in qE (Fig. 4B).

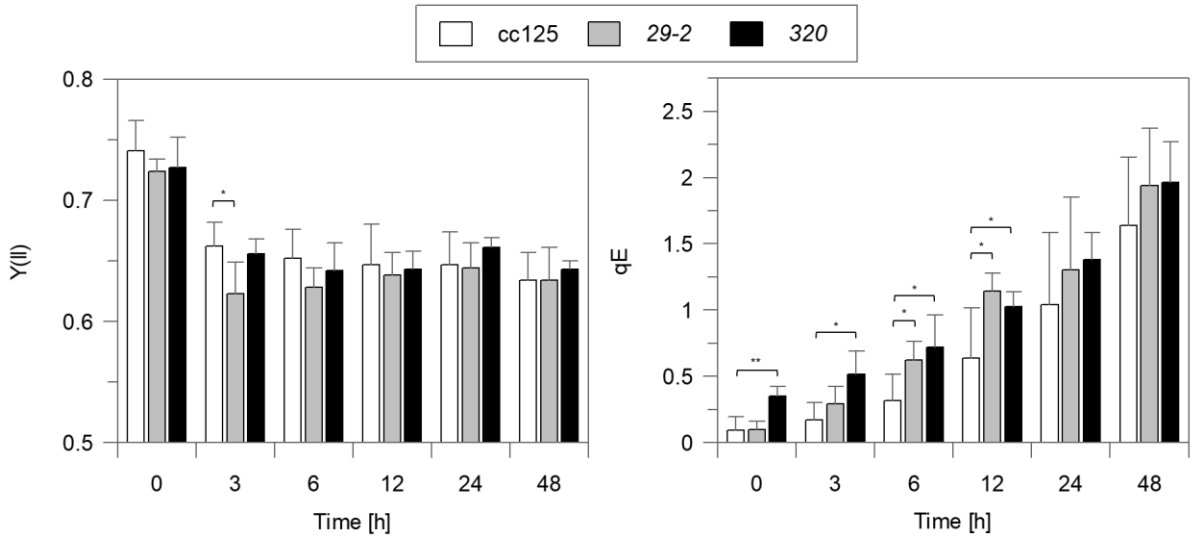

Figure 12: Fitness and stress parameters under fluctuating HL conditions. Quantum yield of photosystem II (A) and qE capacity (B) are shown for WT cc125 and PsbS mutants *29-2* and *320*. Mean values + SD of 3-4 biological replicates are shown. Brackets with asterisks indicate significant differences (*, $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) compared to the wild type.

We further analyzed the accumulation of PsbS and LHCSR proteins during acclimation to fluctuating HL. As expected, no PsbS was detectable in both PsbS knockout mutants (Fig. 5A and S2). In WT cells, however, the accumulation of maximum PSBS amounts occurred more rapidly than under constant HL, whereas the subsequent degradation of PsbS was delayed. The accumulation of LHCSR3 followed similar characteristics as under constant HL and no

significant differences were detectable between WT and mutant cells (Fig. 5B and S2). Regarding the LHCSR1 protein, the mutant lines exhibited a slightly increased accumulation than the WT (Fig. 5C) which might explain the observed increased qE of the mutants (Fig. 4B and S2). In conclusion, the absence of PsbS has no negative impact on HL acclimation and activation of NPQ.

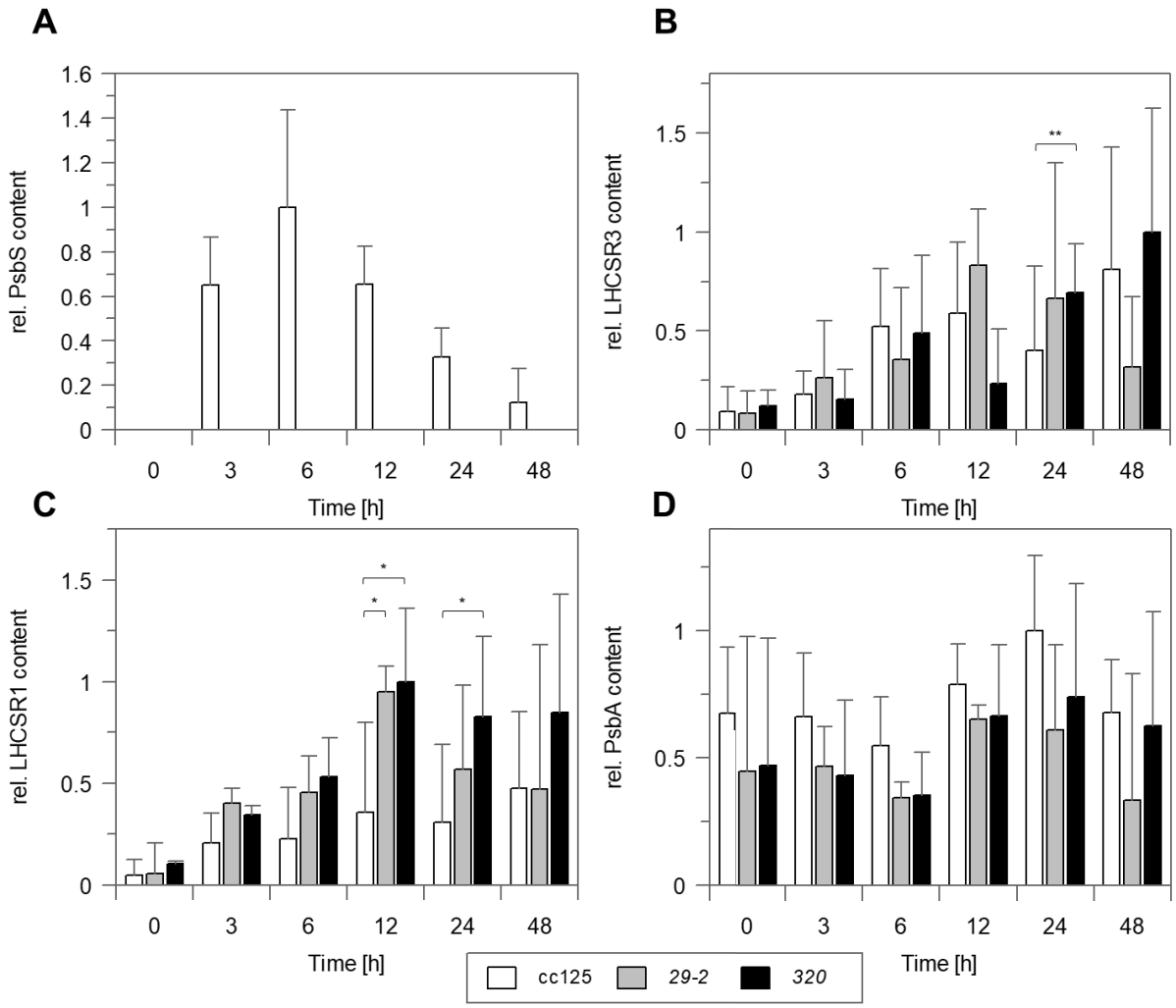

Figure 13: Protein accumulation under fluctuating HL in WT and PsbS mutants *29-2* and *320*. The accumulation patterns of PsbS (A), LHCSR3 (B), LHCSR1 (C) and PsbA (D) are shown. No PsbS was detectable in the mutants at any point and in the WT before HL initiation. Mean values + SD of 3-4 biological replicates are shown. Brackets with asterisks indicate significant differences (*, $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) compared to the wild type.

The most pronounced difference between WT and PsbS-deficient lines was determined for the DEPS of the xanthophyll cycle pigments (Fig. 6A). At any time point during HL exposure, the DEPS of the mutant lines was about 30% lower than in WT cells. However, this reduction was only significant after 24 h of fluctuating HL, due to the rather large standard deviation.

Interestingly, the general VAZ pool size (Fig 6B) grew to similar levels over the illumination period even though it was slightly higher in the wild type, too. The observation of a higher DEPS in the wild type is supported by the accumulation of Ax (Fig. 6C) that has a similar arch as the DEPS. Compared to constant HL conditions, a similar steady-state DEPS of about 40% was determined for WT cells (Figs.1B and 6A). Hence, fluctuating HL thus specifically induces a reduction of the DEPS in PsbS-deficient mutants.

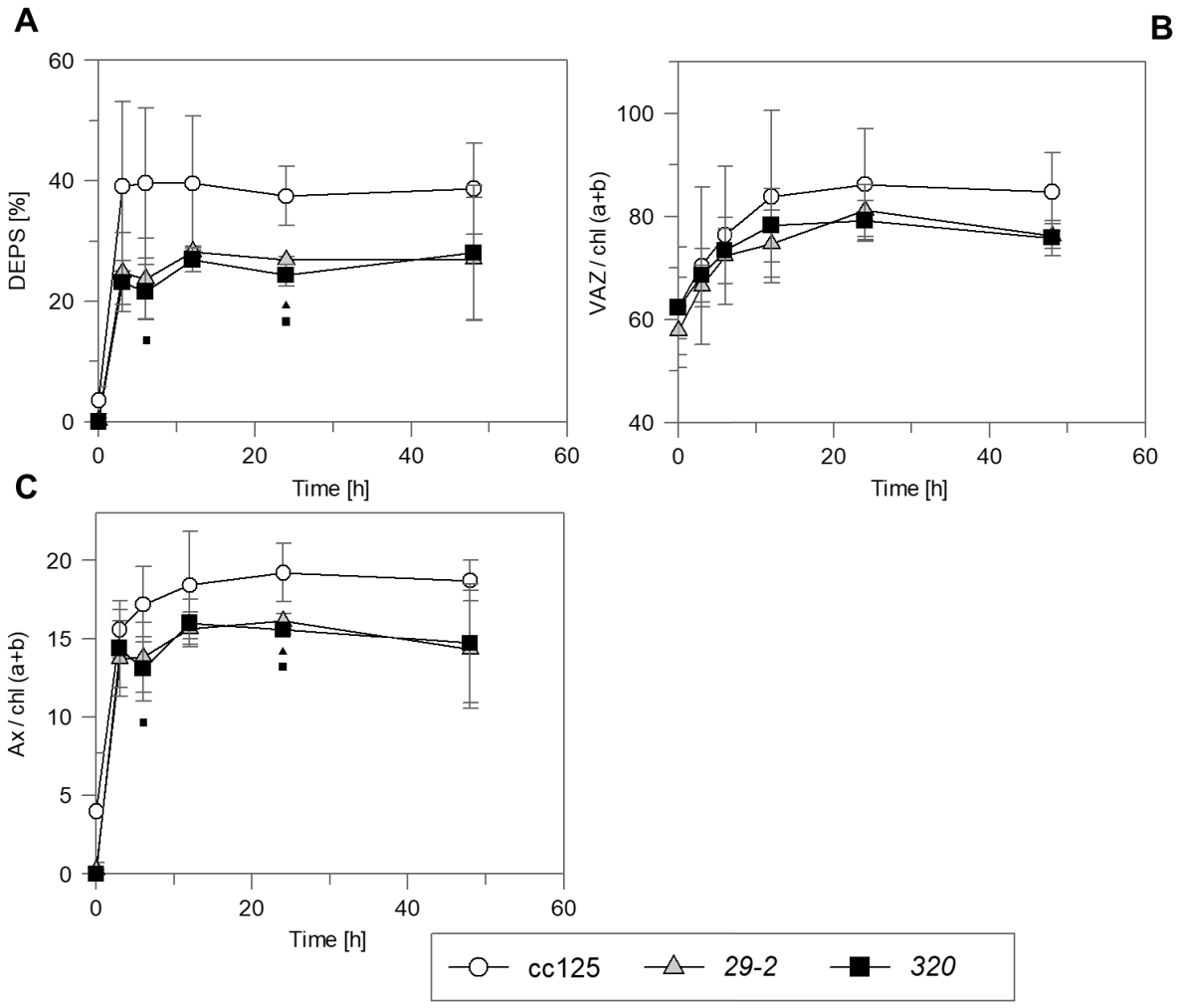

Figure 14: Pigment composition of WT and PsbS-deficient strains under fluctuating high light conditions. The de-epoxidation state of xanthophyll pigments (A), the total VAZ pool size (B) and the amount of the xanthophyll antheraxanthin (C) are shown. Data represent mean values +/- SD of 3-4 biological replicates are shown. Small shapes ($p < 0.05$) indicate significant differences compared to the wild type.

DISCUSSION

The precise role of PsbS in *C. reinhardtii* has been subject to several studies. Because PsbS is the main player in qE in higher plants and because PsbS from vascular plants and *C. reinhardtii* are similar in structure, it has been assumed that PsbS might play a role in qE in *C. reinhardtii* as well (Anwaruzzaman et al., 2004). This speculation was supported by the observed transient accumulation of PsbS during acclimation of Chlamydomonas cells to constant HL (Tibiletti et al. 2016, Correa-Galvis et al. 2016), but the absence of PsbS after about 24 h of HL acclimation, when NPQ is fully activated, argues against a central function of PsbS in pH regulation of qE like in higher plants. However, the reduction of the LHCSR3 amount alongside the reduction of NPQ in PsbS knockdown lines suggested a possible role of PsbS in the reorganization of the PSII antenna during the establishment of LHCSR3-dependent NPQ (Correa-Galvis et al. 2016). Nevertheless, PsbS over-expressing cells had no strong impact on LHCSR accumulation and NPQ capacity, giving rise to the speculation that PsbS might serve photoprotective functions independently from NPQ (Redekop et al. 2020). Such an NPQ-independent function is supported by recent work studying the dynamics of PsbS accumulation under FL conditions (manuscript I) and in response to combined salt and HL stress (manuscript II). To clarify the role of PsbS in *C. reinhardtii*, it is essential to study mutants, in which both *PSBS* genes are completely knocked out. Here, we present the first analysis of two PsbS knock-out strains in comparison with wild type cc125 under constant and fluctuating high light conditions. Strikingly, we found only minor differences among WT and mutant cells with respect to NPQ induction and LHCSR protein accumulation. In particular, the reduction of qE and LHCSR3 accumulation that has been reported earlier for *PSBS* knockdown lines (Correa-Galvis et al 2016), was not confirmed with *PSBS* knockout lines under both constant HL (Figs 1 and 2) and fluctuating HL (Figs 4 and 5) conditions. This unexpected result can most likely be explained by secondary effects of the artificial microRNA induced silencing of the two *PSBS* genes on LHCSR3 expression in the former work. Independent of this discrepancy, the absence of any negative impact of the knockout of both *PSBS* genes on qE strongly argues against any role of PsbS in the establishment of NPQ.

Earlier work further proposed a photoprotective function of PsbS independently of NPQ (Redekop et al. 2020). To address this aspect, we determined the growth and the quantum yield of PSII, Y(II). However, none of these parameters showed significant differences between WT and mutant cells under both constant and fluctuating HL conditions. Obviously, PsbS has no photoprotective function that affects photosynthetic performance and cell growth under our experimental growth conditions. But it has to be noted, that application of other stress conditions might unravel a so far unknown function of PsbS. The performance of the PsbS knockout mutants should particularly studied under conditions that are known to increase PsbS expression or to alter the degradation of PsbS, including UV-B stress (Allorent et al. 2016), low

CO_2 (Correa-Galvis et al 2016), growth under light/dark fluctuations (manuscript 1) or combined HL and salt stress (manuscript 2).

The only parameter that was found to be clearly affected in the PsbS knockout mutants was the DEPS of the xanthophyll cycle pigments, which was reduced in the mutants compared to the WT (Fig. 6), implying a reduced synthesis of Zx in absence of PsbS. In general, the extent of Zx formation depends on the activities of the two xanthophyll cycle enzymes, Vx de-epoxidase (VDE) and Zx epoxidase (ZEP), and on the accessibility or convertibility of the substrates, Vx and Zx. In vascular plants, the VDE is localized in the thylakoid lumen and its activity is strictly regulated by the lumen pH (Bratt et al. 1995; Hager and Holocher, 1994) In *Chlamydomonas*, however, the VDE is localized in the stroma, but nevertheless requires the build-up of a pH gradient across the thylakoid membrane for activity (Li et al. 2016), indicating that the VDE activity in Chlamydomonas is also regulated in dependence of the saturation of the photosynthetic electron transport. Based on the unaffected Y(II) and the similar qE characteristics, it is reasonable to assume that electron and proton transport in the mutant lines is similar than in WT cells. Assuming that the amounts of VDE and ZEP are similar in WT and mutant cells, a possible explanation for the reduced DEPS in the PsbS knockout lines might be an altered availability or convertibility of Vx (or Zx). For higher plants, it is known that binding of Vx to antenna proteins limits the convertibility of Vx to Zx and thus the maximum DEPS (Färber et al. 1997; Jahns et al. 2001). However, the identical maximum DEPS values in mutant and WT cells after 3 h exposure to constant HL (Fig. 3) imply that the convertibility or availability of Vx is not generally affected. Since the DEPS was significantly reduced under fluctuating HL exposure (Fig. 6), it is thus reasonable to assume that particularly fluctuating light conditions alter the conversion of the xanthophylls in absence of PsbS. It remains to be investigated whether Vx to Zx conversion by VDE or Zx to Vx conversion by ZEP is affected under these conditions. Irrespective of that detail, however, it can be speculated that altered membrane properties might be responsible for these characteristics. In land plants, PsbS is supposed to reorganize the PSII antenna during qE activation (Horton et al 1996). Indeed, a role of PsbS in thylakoid membrane organization has also been proposed for Chlamydomonas (Redekop et al 2020), though without any impact on qE. It is thus conceivable that PsbS is required to allow rapid reorganization of the thylakoid membrane in response to fluctuating HL conditions. This reorganization of the membrane might affect the xanthophyll conversion and thus the DEPS. This interpretation agrees with the conclusions derived from recent work on combined HL and salt stress (manuscript 2), which suggested a critical function of PsbS in the re-organization of thylakoid membrane proteins during HL acclimation as well. Interestingly, the increase of the PsbS protein amount under combined HL and salt stress was paralleled by an increase of the DEPS (manuscript 2). Likewise, overexpression of PsbS in tobacco resulted in

a higher DEPS (Hieber et al., 2004), supporting the view that the PsbS amount in the thylakoid membrane may affect xanthophyll conversion.

In conclusion, our analysis of *C. reinhardtii* does not corroborate any important function of PsbS in qE activation during HL acclimation. Instead, a possible photoprotective role might be rather related to a function of PsbS in the organization of the thylakoid membrane.

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SUPPLEMENTARY DATA

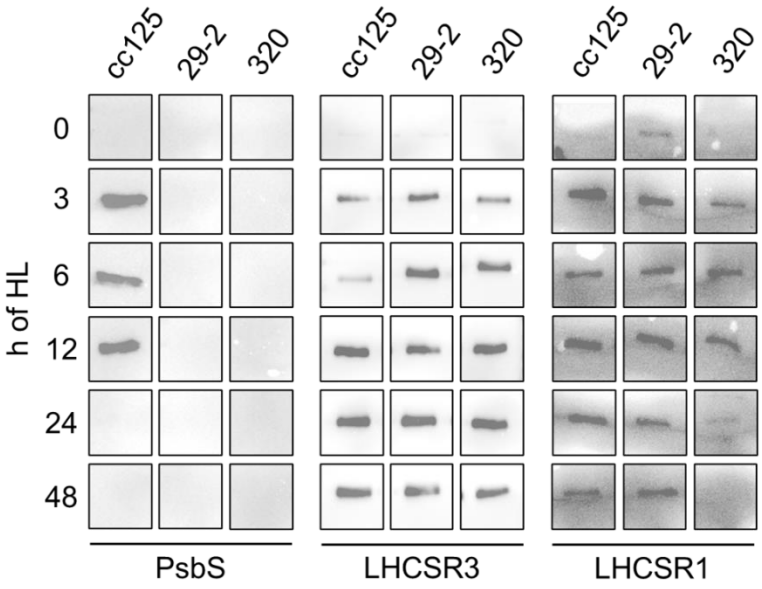

Figure S1: Protein accumulation in constant high light. The accumulation of PsbS, LHCSR3 and LHCSR1 in the wild type cc125 and in the PsbS knock-out mutants *29-2* and *320* was analyzed at three timepoints. Exemplary Western blot images of three biological replicates.

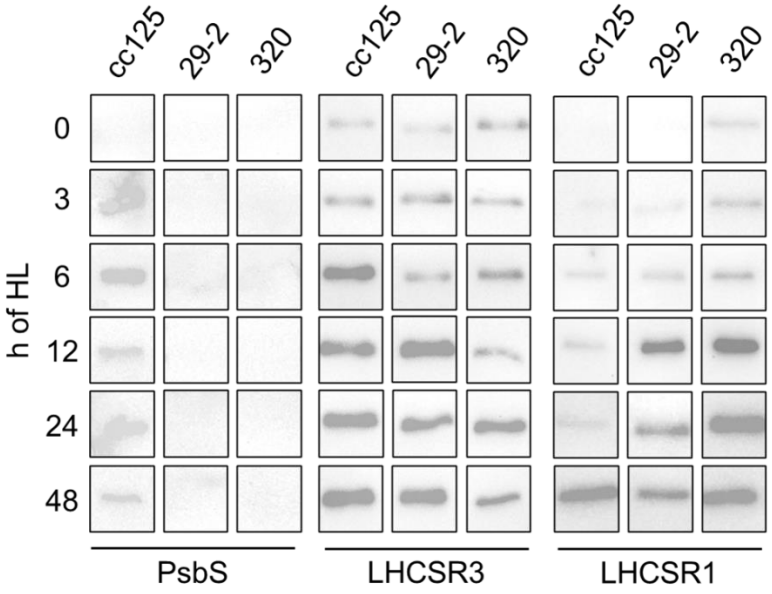

Figure S2: Protein accumulation in fluctuating high light. The accumulation of PsbS, LHCSR3 and LHCSR1 in the wild type cc125 and in the PsbS knock-out mutants *29-2* and *320* was analyzed at three timepoints. Exemplary Western blot images of three to four biological replicates.

9. CONCLUDING REMARKS

C. reinhardtii is a unicellular green alga which promises to serve as a biotechnical production platform of biomass, valuable enzymes, and molecular hydrogen. The advantages of *C. reinhardtii* include a short doubling time and efficient photoautotrophic or mixotrophic growth. To further enhance the productivity of *C. reinhardtii*, it is crucial to understand the processes regulating light utilization efficiency, including efficient photosynthesis under limited light and efficient photoprotection under saturating light. *C. reinhardtii* has served as a model organism for photoprotection for years and its photoprotective strategies are better understood than in any photosynthetic organism aside from higher plants and cyanobacteria. Photoprotection in green algae differs in some crucial elements and offers insights into the evolution of photosynthetic organisms. An especially striking feature is the very different regulation and function of the photoinduced PsbS protein in the photoprotective network that was studied in the present thesis. In the following, some important aspects that have yet to be analyzed are introduced. The present work clarified that PsbS does not have an immediate influence on qE, as it was also speculated before (Redekop et al., 2020). Instead, it is likely that PsbS influences the thylakoid membrane fluidity which affects photosynthesis and photoprotection at different levels. This might also be the reason for the significant increase in accumulation of PsbS under salt stress conditions where membrane properties are of special importance (manuscript 2). Analysis of the lipid composition of the thylakoid membrane in the PsbS knock-out strains is currently ongoing. It is possible that the altered thylakoid membrane fluidity has an influence on the de-epoxidation status of xanthophyll pigments under high light stress conditions (following section I). As this was distinct in fluctuating light conditions, other varying light rhythm conditions might enhance the effect of PsbS and provide further pieces of information on its function and regulation (following section II). Another promising approach to confirm the function of PsbS is to analyze interaction partners as it is likely that PsbS interacts with PSII or LHCII subunits (following section III). In addition, the quantification of PsbS in relation to other photoprotective proteins or PSII antenna proteins might offer insights into its role in photoprotection (following section IV). Aside from the function of PsbS, the mandatory degradation in the high light has not been fully understood, too. The present thesis clarified that the degradation of PsbS during high light acclimation is impaired under fluctuating light conditions with intermediate dark phases (manuscript 1). This underlines that the degradation is actively light driven. Nonetheless, unanswered questions remain as neither the mechanism of degradation nor the necessity for degradation are known, yet (following section V). Clarification of the elusive role of PsbS under stress conditions would be key to understand not only photoprotection in *C. reinhardtii* but also to gain more insight into the evolution of

photoprotection. Another interesting aspect, which has not been clarified so far, is the possible presence of PsbS dimers and a potential light-dependent shift between the monomeric and dimeric form of PsbS as known from vascular plants (following section VI).

I. Influence of PsbS on the xanthophyll cycle

A potential influence of PsbS on the xanthophyll cycle has been proven but it remains unclear whether this is based on a direct or an indirect role of PsbS. In the PsbS knock-out mutants, the DEPS was lower compared to the wild type in constant and especially fluctuating light (manuscript 3). In salt stress conditions (manuscript 2), as well as in fluctuating light conditions with intermittent dark phases (manuscript 1), the DEPS was higher than in control conditions. At the same time, in both cases, the rise in DEPS was accompanied by an elevated PsbS accumulation. It cannot be excluded that PsbS has a direct regulative function on VDE or ZEP activity, but it is probably more likely that the change in membrane fluidity mediated by PsbS is responsible for the altered DEPS. In tobacco, over-expression of PsbS was accompanied by a higher DEPS, too (Hieber et al., 2004). PsbS itself does not bind any pigments (Dominici et al., 2002), so any change in pigment composition cannot be attributed to binding by PsbS. It is possible that PsbS induces the binding of Zx to the quenching complex, which would shift the equilibrium of the xanthophyll cycle pigments towards Zx (Hieber et al., 2004). Similar to tobacco, PsbS over-expressing lines in *C. reinhardtii* might thus have an elevated DEPS, too. In addition, it is at least conceivable that the amount of PsbS affects the kinetics of de-epoxidation or epoxidation. A detailed analysis of the xanthophyll conversion kinetics in wild-type and PsbS mutant lines of *C. reinhardtii* is thus required. Given that PsbS alters the membrane fluidity, it is possible that this affects the binding of VDE to the membrane (Jahns et al., 2009). This could explain why the de-epoxidation state is lower in PsbS knockout lines compared to the wild type. It is reasonable to study the impact of different PsbS amounts on the binding of the VDE and ZEP to the thylakoid membrane.

II. Analysis of PsbS mutants under various stress conditions and diverse light regimes

Most studies concerning photoprotection in *C. reinhardtii* have been conducted in response to a shift of cells from constant low light to constant high light. *C. reinhardtii* preferentially lives in small ponds and puddles where light availability often varies significantly due to clouds or shadowing by trees. In general, fluctuating light rhythms are more natural than constant high light conditions. This thesis has shown that PsbS dynamics are substantially different in fluctuating light conditions, especially with intermittent dark phases (manuscript 1). In addition, PsbS knock-out mutants show a stronger phenotype under fluctuating light conditions

compared to constant light (manuscript 3). This indicates that PsbS is likely more impactful under conditions that are different from constant high light. PsbS might be particularly important for modification of the membrane fluidity at the onset of high light defense, for example by supporting Vx to Zx conversion which might be crucial under fluctuating light conditions. Under the applied fluctuating light (0.5 h HL/1 h dark) conditions, PsbS degradation was almost stopped (manuscript 1). It might be interesting to identify light rhythms under which PsbS degradation is completely. Related to the characteristics of PsbS degradation, it would be possible to learn more about the underlying degradation mechanism. Applying those conditions to PsbS over-expressing or knock-out lines might help to further understand the role of PsbS. The same might apply to the analysis of other stress conditions that affect PsbS accumulation, such as salt stress (manuscript 2), UV light (Allorent et al., 2016) or varying CO_2 availability (Redekop et al., 2020). It will further be interesting to study the impact of drought stress on photoprotection, PsbS and xanthophyll conversion. Drought stress has not only similarities to salt stress but has been shown to upregulate PsbS and NPQ in *Arabidopsis* (Chen et al., 2016). As *C. reinhardtii* is able to grow on solid media, too, it is possible to induce drought stress in a simple way with most *C. reinhardtii* strains.

III. Identification of PsbS interaction partners

PsbS accumulates upon HL exposure and is degraded after several hours in constant HL. In the thylakoid membrane, it is likely that PsbS interacts with other proteins or structures to modify thylakoid membrane organization or structure. Analysis of interaction partners can therefore be expected to provide valuable information not only about the function of PsbS, but possibly also about the degradation of PsbS. In preliminary experiments, the identification of possible interaction partners was addressed by chemical cross-linking of isolated thylakoids of *C. reinhardtii* with three different cross-linkers, namely ethylene glycol bis(succinimidyl succinate) (EGS), 3,3'-Dithiobis(sulphosuccinimidyl propionate) (DTSSP) and m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (sulfo-MBS). EGS is a membrane-permeable amine-to-amine cross-linker with a 16.1 Å cleavable spacer arm (Oh-oka et al., 1989). DTSSP is a water-soluble amine-to-amine cross-linker with a 12.0 Å cleavable spacer arm (Correa-Galvis, Poschmann, et al., 2016). Sulfo-MBS is a water-soluble heterobifunctional cross-linker of amines and sulfhydryl groups with a spacer arm of 7.3 Å (Benz et al., 2009). By applying different cross-linkers, the chance of successful cross-linking was enhanced. After cross-linking, the thylakoid protein complexes were separated by sucrose density centrifugation and by that to obtain information about possible interaction partners of PsbS. Covalently cross-linked PsbS to other proteins should result in the occurrence of additional Western blot bands besides the PsbS monomer band at 22 kDa. The result is exemplarily

shown in Figure 10 for the cross-linker EGS. The individual fractions were analyzed by mass spectrometry to identify crosslinks of PsbS with other proteins. Unfortunately, the detection of PsbS peptides by mass spectrometry was unsuccessful, likely due to the low amounts of PsbS. To further purify and enrich the cross-link products, PsbS was immunoprecipitated. However, the amount of PsbS in samples was still not sufficient for mass spectrometry analysis. To overcome these problems, two further approaches were initiated: (1) Generation of mutants that carry the genetic information for a fusion protein of PsbS and biotin ligase TurboID to have an additional option for identification of PsbS interaction partners. (2) Generation of *C. reinhardtii* lines with recombinant, epitope-tagged PsbS to simplify and enhance purification of cross-linked PsbS and interaction partners. As a common epitope tag in *C. reinhardtii*, human influenza hemagglutinin (HA) tag was chosen (Silflow et al., 2001). Both approaches, identification of biotinylated proteins via mass spectrometry and chemical cross-linking followed by affinity purification of HA-tagged PsbS are promising for identification of interaction partners. In a similar approach applying chemical cross-linking, PsbS interaction partners were identified in *A. thaliana* (Correa-Galvis, Poschmann, et al., 2016). In plants, PsbS interacts with PSII and LHCII proteins as the main qE trigger, but it is possible that interaction partners in *C. reinhardtii* differ, as the function differs substantially, too. As PsbS is degraded in *C. reinhardtii*, it is also interesting to identify proteases or other proteins involved in the process of PsbS degradation (see also below, section V). Since only the net degradation of PsbS is detectable by immunoblot analyses, it would be straightforward to determine in detail the lifetime of PsbS under the different experimental conditions, e.g. by radioactive labelling. This would clarify whether the degradation or the synthesis of PsbS are specifically regulated in response to the different stress conditions.

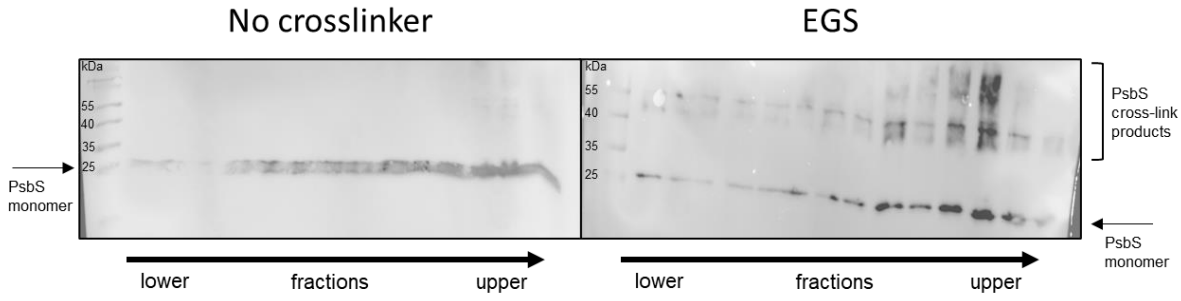

Figure 15: Immunoblot analysis of sucrose density gradient fractions with and without cross-linker EGS. The thylakoid membranes of a PsbS over-expressing line were isolated. The thylakoid membranes were cross-linked with EGS and subjected to sucrose density gradients. The fractions were analyzed by immunoblotting with the PsbS antibody. When compared to the negative control without cross-linking, multiple bands with higher molecular mass were detected in the EGS-treated samples indicating successful cross-linking of PsbS. At 22 kDa, the PsbS monomer was detected in most fractions.

IV. Quantification of PsbS

In addition to determining potential interaction partners, quantification of PsbS in relation to PSII, LHCII and LHCSR proteins is important. Together with the interaction studies, such data would provide a solid basis for the understanding of the function and regulation of PsbS. At the same time, the LHCSR proteins are yet to be quantified in *C. reinhardtii*, too. Assuming a similar affinity and reactivity of the used antibodies, it can be estimated from immunoblot analyses, that PsbS accumulates in wild-type cells to only substoichiometric amounts in relation to PSII and LHCII even under severe high light stress, while LHCSR proteins likely accumulate to rather similar stoichiometries as PSII and LHCII. Exact quantification of PsbS and LHCSR proteins would provide valuable information to understand the reorganization of the PSII antenna during HL acclimation. The quantification could ideally be accomplished by mass spectrometry using isotopically labeled whole cell extracts in conjunction with purified recombinant standard proteins (Nikolova et al., 2017). Obtaining precise stoichiometries of PsbS in comparison to potential interaction partners would substantially improve the understanding of PsbS function.

V. PsbS Degradation

The degradation of CrPsbS is a very striking feature, especially when compared to higher plants, where PsbS is constitutively expressed and not degraded in high light. In *C. reinhardtii*, degradation is obligatory in constant high light and occurs not only in the wild type but also in PsbS over-expressing lines (Redekop et al., 2020). However, it remains unknown so far, why the degradation of CrPsbS is mandatory. Up to this point, no negative impact of PsbS has been identified, neither under LL nor HL conditions. PsbS over-expressing strains did not show any obvious disadvantage with respect to growth, fitness or photoprotection. Moreover, PsbS degradation does not correlate with an enhancement of qE or an activation of other high light defensive strategies. It has been speculated that PsbS might interfere with LHCSR binding sites and prevent full activation of qE (Redekop et al., 2020). In the present thesis, however, no evidence of a connection between qE and PsbS availability was detected, but PsbS degradation was almost stopped in fluctuating light with intermittent dark phases (manuscript 1). This implies that PsbS degradation is a light-dependent process. The D1 turnover is another long-term high light acclimation strategy in which a protein of the thylakoid membrane is degraded. This has led to the assumption that the degradation of D1 and PsbS might be conducted by the same proteases. To test this hypothesis, protease-deficient mutants of *C. reinhardtii* were analyzed with respect to PsbS accumulation pattern (Rothhausen, 2018) (Fig. 10). To this end, knock-out mutants of degradation of periplasmic proteins/high temperature

required A (DegP/HtrA) proteases (referred to as Deg proteases) and filamentation temperature sensitive H (FtsH) proteases were analyzed, as both protease groups are involved in D1 turnover in the chloroplast (Adam et al., 2005; Kapri-Pardes et al., 2007; Malnoë et al., 2014). *Arabidopsis* possesses 16 genes encoding Deg proteases, eight of which have been detected in the *C. reinhardtii* genome (Huesgen et al., 2005). Concerning Ftsh proteases, twelve genes were described in *Arabidopsis* with *C. reinhardtii* possessing only two homologs (Sakamoto et al., 2003). As shown in Fig. 10, mutants devoid of Deg1, Deg5, Deg8 and Ftsh1 were identified to be impaired in PsbS degradation in comparison with the wild type. In contrast, an Ftsh2 knock-out mutant showed the same pattern of PsbS degradation as the wild type, arguing against a role of Ftsh2 in PsbS degradation. Deg1, Deg5 and Deg8 are known to be located in the thylakoid membrane (Schuhmann et al., 2012). Ftsh1 and Ftsh2 are both upregulated upon HL incubation and accumulate with photooxidative stress (Barth et al., 2014). Therefore, both protease groups are promising candidates for the degradation of PsbS. This could also be verified by the analysis of interaction partners of PsbS (see section III).

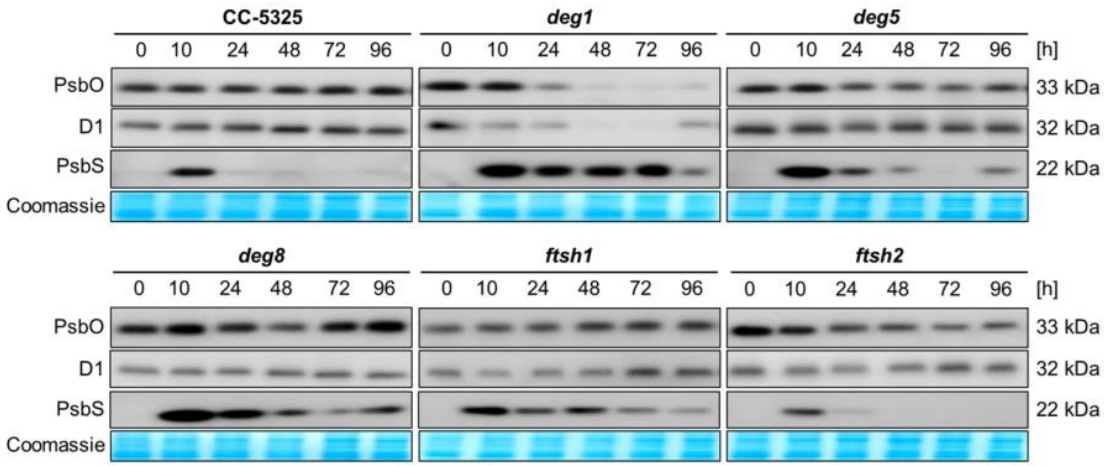

Figure 16: Protein accumulation in protease-deficient mutants. The accumulation of PsbO, D1 and PsbS are shown over a time span of 96 h under HL of 350 µE. Representative images of three independent replicates are shown. From:(Rothhausen, 2018).

The regulation of PsbS degradation is another matter for discussion. Mutants affected in other HL acclimation than qE, such as *stt7* (Depège et al., 2003), *pgrL1/pgr5* (Kukuczka et al., 2014) and *cas* (Petroutsos et al., 2011) were analyzed with respect to PsbS accumulation during HL acclimation (Westhäuser, 2021). Strikingly, no PsbS degradation was detectable in *stt7* and *cas* mutants even after 48 h of high light exposure (Fig. 12), indicating that PsbS degradation is part of a more complex network involving the interaction of different HL acclimation processes. STT7 is a Ser/Thr kinase that is mostly responsible for phosphorylation of LHCII proteins in state transitions (Depège et al., 2003). It is conceivable that STT7 phosphorylates PsbS, which could be a prerequisite for PsbS degradation by Deg or Ftsh proteases.

Alternatively, STT7 might activates kinase STI1 via phosphorylation (Lemeille et al., 2010) which in turn could phosphorylate PsbS. STI1 is an ortholog of STN8 in *Arabidopsis* which is responsible for phosphorylation of D1 protein during the D1 turnover (Vainonen et al., 2005). Detailed analysis of the phosphorylation state of PsbS during HL acclimation is required to test the possible requirement of phosphorylation for PsbS degradation. Phosphorylation of PsbS might also be triggered by changes in Ca^{2+} levels. CAS is a calcium-sensing protein that has been shown to be involved in high light acclimation (Petroutsos et al., 2011). Possibly, a HL induced change of the Ca^{2+} concentration is not properly sensed in *cas* knock-down mutants, which might result in the missing activation of PsbS degradation (Fig. 11). This hypothesis is supported by the observation of a Ca^{2+} ion flux into the chloroplast stroma during a light to dark transition (Sai & Johnson, 2002), which could then explain why PsbS degradation was suppressed under fluctuating light with intermittent dark phases (manuscript 1).

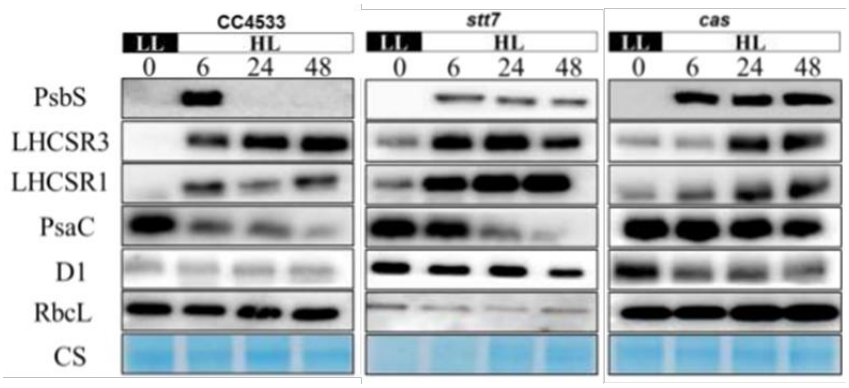

Figure 17: Protein accumulation in mutants affected in HL acclimation. The accumulation of PsbS, LHCSR3, LHCSR1, PsaC, D1 and RbcL was determined by immunoblot analyses for up to 48 h of HL exposure (350 µmol photons m^{-2} s^{-1}). Representative images of four independent replicates are shown. From:(Westhäuser, 2021).

VI. Immunoblot analysis of a potential PsbS dimer

The production of antibodies specific for CrPsbS antibody substantially improved the understanding of PsbS in *C. reinhardtii* (Correa-Galvis, Redekop, et al., 2016; Tibiletti et al., 2016). Applying these antibodies in immunoblot analyses two bands were detected in some cases, one at about 22 kDa and one at about 40 kDa (Fig. 12). The predicted size of CrPsbS is 26 kDa (Uniprot accession: A8HPM2). The first 25 amino acids are predicted to form a chloroplast transit peptide, so the 22 kDa band likely represents the PsbS monomer. The 40 kDa band has been attributed to a putative PsbS dimer (Nawrocki et al., 2020).

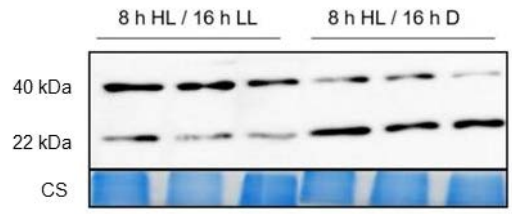

Figure 18: Immunoblot analysis applying the PsbS antibody on whole-protein extracts of *C. reinhardtii* cc124. Two distinct bands per lane are detected, one resembling the PsbS monomer at about 22 kDa and one at about 40 kDa. Three biological replicates are shown, each after 8 h of high light in short-day rhythm with intermittent dark or low light phases. From (Ammelburger, 2022)

Dimeric PsbS is expected to be the inactive form of PsbS. The activation and monomerization is supposed to be mediated by the acidification of the lumen as a result of high light stress conditions. Nonetheless, dimerized PsbS was detected in *Physcomitrella patens in vitro* even at lower pH (Krishnan et al., 2017). In *Zea mays*, a PsbS dimer was also detected *in vitro* without high light stress (Bergantino et al., 2003). In *C. reinhardtii*, there has not been any proof of the existence of a PsbS dimer up to this point. Nonetheless, a recent work postulated that the upper 40 kDa band corresponds to dimeric PsbS and hence used both signals for quantification of PsbS (Nawrocki et al., 2020). To test the hypothesis of the presence of a PsbS dimer in *C. reinhardtii*, several pieces of evidence were collected in the present work that strongly argue against the assignment of the 40 kDa band to a PsbS dimer. Firstly, SDS-PAGE under denaturing conditions with reducing agents like dithiothreitol (as used in the experiment shown in Fig. 13) should impair the interaction of PsbS monomers. Secondly, the 40 kDa band is detectable even under low light conditions, although PsbS expression is only triggered under light stress conditions (Allorent et al., 2016). Thirdly, the 40 kDa band is quite stable, irrespective of the light-induced increase or reduction of the amount of PsbS monomers. Fourthly, the 40 kDa band is also detectable in PsbS knock-out mutants (Fig. 13). In the experiments with fluctuating light (manuscript 1), a cross-reaction of the LHCSR1 antibody with a band at the same height as the 40 kDa band was observed. Therefore, it was suggested that possibly a heterodimer or an aggregate of PsbS and LHCSR1 might be behind the 40 kDa band. To test this hypothesis, knock-out mutants of PsbS and LHCSR1 were analyzed regarding the 40 kDa band (Fig. 13). Since the 40 kDa band was detectable in both mutants, it can be excluded that both or either protein contributes to the 40 kDa band. Instead, it is likely that another, unknown protein is responsible for the cross-reaction. It is thus crucial to exclude the 40 kDa band for quantification of PsbS.

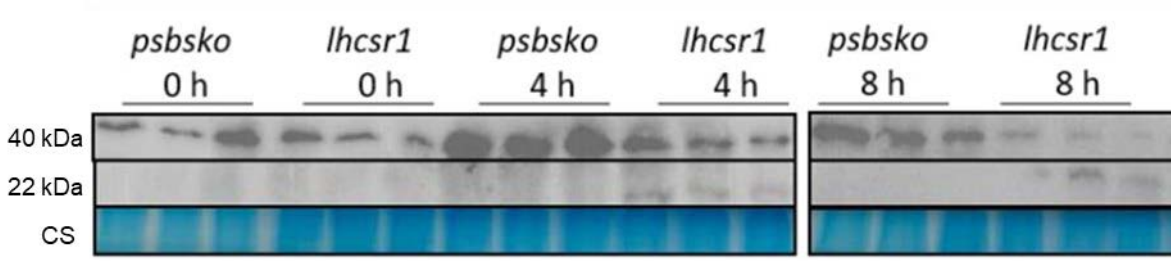

Figure 19: Immunoblots analyses of the PsbS antibody with PsbS knock-out (*psbsko*) and LHCSR1 knock-out (*lhcsr1*) mutants. Cells were grown in low light (0 h) and shifted to high light (4 h, 8 h). PsbS monomers were only detected after 4 h and 8 h in lhcsr1. The 40 kDa band was visible in all samples at all timepoints. From (Ammelburger, 2022)

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