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Enhanced function of non-photoinhibited photosystem II complexes upon PSII photoinhibition

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ABSTRACT

Light induced photosystem (PS)II photoinhibition inactivates and irreversibly damages the reaction center protein(s) but the light harvesting complexes continue the collection of light energy. Here we addressed the consequences of such a situation on thylakoid light harvesting and electron transfer reactions. For this purpose, *Arabidopsis thaliana* leaves were subjected to investigation of the function and regulation of the photosynthetic machinery after a distinct portion of PSII centers had experienced photoinhibition in the presence and absence of Lincomycin (Lin), a commonly used agent to block the repair of damaged PSII centers. In the absence of Lin, photoinhibition increased the relative excitation of PSII and decreased NPQ, together enhancing the electron transfer from still functional PSII centers to PSI. In contrast, in the presence of Lin, PSII photoinhibition increased the relative excitation of PSI and led to strong oxidation of the electron transfer chain. We hypothesize that plants are able to minimize the detrimental effects of high-light illumination on PSII by modulating the energy and electron transfer, but lose such a capability if the repair cycle is arrested. It is further hypothesized that dynamic regulation of the LHCII system has a pivotal role in the control of excitation energy transfer upon PSII damage and repair cycle to maintain the photosynthesis safe and efficient.

1. Introduction

In photosynthetic electron transfer chain (ETC), the thylakoid-embedded photosystem (PS)II, Cytochrome (Cyt) b_6f complex and PSI work in series, and together with the ATP synthase convert light energy into chemical energy. All excitation energy and electron transfer reactions must take place in great synchrony to keep photosynthesis efficient, to minimize damaging side reactions and to cope with the consequences of the damage, and thus are dynamically co-regulated.

Light energy exciting the photosynthetic reaction centers (RC) is absorbed by pigment-binding light harvesting complexes (LHC) [1]. In the light harvesting antenna, the absorbed energy is transferred to the nearest correctly oriented pigment, until it reaches the RC chlorophyll (chl) *a* pair of PSII and PSI – P680 and P700, respectively. The excited P680 donates electrons via pheophytin to a plastoquinone (PQ) molecule, which after two electron transfer reactions is protonated to PQH_2. Reduced PQH_2 donates electrons further to the Cyt b_6f complex that, via plastocyanin, reduces the PSI RC $P700^+$. The generation of either highly-oxidizing or reducing species in photo-catalytic turnover of PSII and PSI, respectively, is potentially dangerous for the pigment–protein complexes and, for this reason, a fraction of PSII RCs is always damaged. In damaged PSII RC, the charge separation is no longer possible and excited chlorophylls must find other paths to relax to prevent the formation of singlet oxygen. Such mechanisms have remained unknown, but in theory, energy can be redirected to still functional RCs via LHCII system or to be dissipated as heat by the inhibited PSII centers.

PSII−LHCII supercomplexes (sc) in plants are composed of a dimeric core, containing the core antenna proteins CP43 and CP47, and variable amounts of trimeric LHCII antennas attached to the core with different affinities. The strongly attached S-LHCII is bound to the core via CP26 (Lhcb5) and the moderately attached M-LHCII via CP24 (Lhcb6) and CP29 (Lhcb4). The S-LHCII is known to be directly connected to CP43, while the excitation energy collected by M-LHCII has been proposed to reach the core CP47 antenna via its connector protein CP29 or via S-LHCII and CP43 [1]. In addition, a large pool of loosely bound L-LHCII is thought to mediate the exchange of excitation energy between the different PSII–LHCII sc as well as to energetically connect the remotely localized PSII and PSI [2,3]. The relative interaction between PSII-LHCII

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complexes and the antenna sizes of PSII and PSI are modulated by reversible phosphorylation of the protein subunits of PSII and LHCII [3–7]. The efficiency of the antenna size is dynamically regulated according to the light intensity and the capacity of the plant metabolism to make use of collected energy. Any excess energy is thermally dissipated by mechanisms generally called NPQ [8], which diminishes the light harvesting efficiency when the amount of light energy received exceeds the plant's capacity to utilize it in metabolism. Research on the LHCII system has mostly focused on regulation of light harvesting under different light intensities and qualities, but it is highly likely that the dynamics of the LHCII system has a pivotal role also in the control of excitation energy transfer upon acclimation to photoinhibition [9].

PSII is extremely sensitive to excess light energy and is, for this reason, constantly damaged with a rate that is linearly dependent on light intensity [10,11]. The molecular mechanisms operating in the LHCII system and leading either to generation of PSII photodamage or to protection against such a damage via dissipation of excess energy by NPQ, still remain under discussion. There is, however, evidence that a carotenoid-dependent protection of PSII against photoinhibition takes place in PSII-LHCII sc via quenching of triplet chlorophylls [12]. Nonetheless, plants have developed an efficient and dynamically regulated repair cycle that maintains the PSII activity as long as the rate of photodamage does not exceed the rate of repair [13]. The sacrifice of mostly only one protein, the D1 RC protein, seems to protect the rest of PSII proteins and surrounding biomolecules from wider damage [13]. To be able to specifically address the PSII damaging process, a common approach has been to use chloroplast translation inhibitors to arrest the repair cycle. These inhibitors have enabled detailed investigation of the damage process, but their side effects on the structure and function of the photosynthetic machinery have remained unclear.

Managing excitation energy transfer during the PSII damage and repair cycle requires high flexibility from the LHCII system, yet it is not known how the excitation energy collected by the LHCII system is safely handled upon PSII photoinhibition and the subsequent repair process. The repair cycle is a dynamic process including both the disassembly and subsequent reassembly of PSII complexes, migration of PSII subcomplexes between appressed grana stacks and stroma- exposed thylakoid domains as well as the synthesis and insertion of the de novo synthesized D1 protein into the PSII subassembly [14]. The unpacking of damaged PSII is facilitated by the STN8 kinase-dependent phosphorylation of PSII core proteins [15–17], while the degradation of D1 protein requires dephosphorylation [18] that is, at least partially, under the control of the PBCP phosphatase [19]. PSII core protein phosphorylation together with STN7 kinase and TAP38/PPH1 phosphatase-dependent reversible phosphorylation of LHCII proteins, are involved in the regulation of energy distribution between PSII and PSI [5,20,21]. The role of thylakoid protein phosphorylation in regulation of excitation energy distribution during the PSII damage and repair cycle has not been previously investigated.

As frequently reviewed [14,22–25], the PSII repair cycle comprises complicated and time-wise highly coordinated mechanisms, which always keep a certain fraction of PSII in damaged state or under different steps of the repair process, thus being photochemically inactive. Moreover, the consequences of PSII photodamage not only concern the loss of active PSII RCs and decrease in linear electron transfer, but the repair cycle also has a high energy demand [22,26]. For this reason, it is conceivable that plants need efficient mechanisms to optimize the rate of light reactions– not only to compensate the decrease in the number of active RCs but also to overcome the increased energy demand.

Here we addressed the impact of PSII photoinhibition, induced in the presence and absence of chloroplast translation inhibitor lincomycin (Lin), on excitation energy and electron transfer in the thylakoid membrane. Results indicate that plants have a high capacity to compensate the decrease in the number of active PSII RCs by enhancing the light harvesting and electron transfer of the still functional PSII RCs. These mechanisms were mostly abolished by the presence of Lin, which segregates the PSII damage and protein degradation reactions from the repair cycle of PSII, suggesting that the results obtained by using chloroplast translation inhibitors might differ from natural PSII photoinhibition.

2. Results

2.1. Modulation of PSII to PSI stoichiometry and energy distribution from the light harvesting antenna to photosystems upon PSII photoinhibition

Photoinhibition of PSII induces a strong quenching of chl fluorescence and is often monitored as a decrease in the F_V/F_M parameter, which estimates the maximal quantum yield of PSII [27,28]. Here, we followed the decrease of F_V/F_M and associated events in *Arabidopsis thaliana* leaves incubated overnight in water and in water supplemented with 1 mM Lin in darkness, and subsequently shifted to HL (1000 μmol photons m^{-2} s^{-1}) for 0.5 and 1 h to induce different levels of PSII photoinhibition (Fig. 2). Dark acclimated control leaves showed a F_V/F_M value of about 0.81, which is typical for healthy, non-photoinhibited leaves (Fig. 2A) [29]. The HL treatment in water decreased the F_V/F_M value to 0.73 during the first 0.5 h and to 0.66 during the entire 1 h treatment, indicating about 10 % and 20 % loss in PSII function. In the presence of Lin, Fv/ F_M decreased to 0.67 in 0.5 h and to 0.5 in 1 h HL treatment, indicating about 20 % and 40 % loss in PSII function, respectively (Fig. 2 A).

Since the F_V/F_M parameter is affected not only by quenching of F_M, but also by changes in F_0 ($F_V = F_M–F_0$), the F_0 and F_M variables are presented also separately in Fig. 2. In line with earlier reports [30,31], the F_M value decreased similarly to F_V/F_M as a response to HL in the presence and absence of Lin (Fig. 2B), contributing to the decrease in F_V/F_M. Contrary to the decrease in F_M, the F_0 value increased in the course of the HL treatment, with the F_0 increase being proportional to the decrease of F_M, both in the presence and absence of Lin (Fig. 2C). Decrease in F_M observed after 0.5 and 1 h HL treatments indicated that the number of RCs getting closed by a saturating pulse decreased as photoinhibition progressed. Concomitant increase in F_0, on the other hand, demonstrated enhanced background fluorescence emission, which could be derived either from the light harvesting system detached from functional PSII RCs, from damaged PSII RCs or from PSI. The number of PSI RCs with photo-oxidable P700 (P_M) remained rather unaffected during the HL treatments (Fig. 2D), yet some decrease was detectable with and without Lin after 1 h HL treatment, probably due to minor loss of FeS centers accepting electrons from P700 and enabling the oxidation of PSI [32].

To investigate the impact of PSII inhibition on transfer of excitation energy between the thylakoid pigment-protein complexes, we analyzed the composition of thylakoid protein complexes by blue native gel electrophoresis (BN-Gel) and monitored the 77 K fluorescence emission spectra as well as the phosphorylation status of thylakoid proteins. When the thylakoid protein complexes were analyzed by BN gel electrophoresis (Fig. 3), it was clear that in HL in the presence of Lin, the PSII sc were partially disassembled indicating that PSII sc with photoinhibited PSII RCs were disassembled, but not repaired due to the translation inhibitor Lin. Disassembly of PSII sc inevitably changes the excitation energy transfer between PSII-LHCII complexes and from LHCII trimers to PSI. Moreover, in the absence of Lin a faint PSI-LHCII complex was detected in LL indicating efficient LHCII excitation of PSI, but in the presence of Lin the complex disappeared.

Next, the impact of PSII photoinhibition on the excitation energy transfer in the thylakoid membrane was assessed by recording the 77 K fluorescence emission spectra (Fig. 4). No clear shifts in peaking of the emission bands, indicative of detached or aggregated LHCII, were recorded in the absence or presence of Lin. This suggested that the energy transfer from LHCII trimers to PSII and PSI remained undisturbed. In the presence of Lin, in line with the decrease in F_M (Fig. 2 B), the relative PSII to PSI fluorescence decreased after 1 h HL treatment and

this was visible after both the 0.5 h LL and EL treatments (Fig. 4), indicating that the decreases in F_M and relative PSII fluorescence at 77 K might result from the same photoinhibition associated quenching process. In the absence of Lin, the relative PSII to PSI fluorescence emission did not decrease despite a clear decrease in F_M, indicating that the mechanism behind the decrease in F_M in vivo does not occur in isolated thylakoids at 77 K. When leaves were exposed to LL in the absence of Lin, relative PSII to PSI fluorescence showed some variation depending on whether the leaves were moved to LL from darkness or from 0.5 or 1 h in HL (Fig. 4 A) and the changes correlated with the visibility of the PSI-LHCII complex (Fig. 3). In HL (Fig. 4 C), in turn, the relative excitation remained unchanged independently of the light treatment, and PSI-LHCII complex was not detected (Fig. 3).

Since it is well known that, from one hand, the excitation energy distribution from LHCII to PSII and PSI (Fig. 4) is regulated by phosphorylation of the LHCII proteins Lhcb1 and Lhcb2 [3] and, from the other hand, the unpacking in grana of the PSII sc after PSII photoinhibition is facilitated by phosphorylation of the PSII core proteins D1, D2 and CP43 [16,17,33], we next applied P-Thr antibodies to immunodetect these thylakoid phosphoproteins. To be able to compare the dynamics between D1 degradation and changes in phosphorylation, the same thylakoids isolated from leaves treated as explained in Fig. 1 were used for immunoblotting. The amount of the D1 protein gradually decreased during the HL treatment in the presence of Lin but not in the absence of Lin (Fig. 5). In the absence of Lin (-Lin), the phosphorylation of PSII-LHCII proteins of 0.5 and 1 h HL treated leaves showed typical response to different light intensities, suggesting that the PSII capacity to reduce the PQ pool and the stromal redox component regulating the thylakoid protein kinases, was not significantly affected despite the decrease in F_V/F_M (Fig. 2). In LL, the relative phosphorylation level of PSII core proteins was low in relation to the phosphorylation of LHCII, and in HL, the phosphorylation of PSII core proteins increased and the phosphorylation of LHCII decreased. In the presence of Lin (+Lin), the behavior of Lhcb1 + Lhcb2 protein phosphorylation changed drastically. Hardly any phosphorylation of LHCII proteins took place in LL nor in EL. Even in 0.5 h HL treated leaves the LHCII proteins were not phosphorylated, suggesting that limited PSII electron transfer led to increased oxidation of ETC and consequent inactivation of the STN7 kinase. The striking difference between the 0.5 h + Lin and 1 h -Lin experiments, however, indicated that Lin has an additional impact on regulation of the STN7 kinase.

2.2. *Consequences of PSII photoinhibition on photosynthetic electron transfer chain*

PSII photoinhibition enhances the function of non-inhibited PSII centers.

The HL treatment decreased Y(II) in LAL but not in EAL (Fig. 6A). In LAL, the Y(II) value decreased from 0.70 to 0.67 during the first 0.5 h and to 0.60 during the entire 1 h treatment, indicating about 5 % and 15 % loss in PSII yield. In the presence of Lin, Y(II) decreased from 0.69 to 0.64 in 0.5 h and to 0.45 in 1 h HL treatment, indicating about 7 % and 35 % loss in PSII function, respectively (Fig. 6 A). The impact of HL treatment was smaller on Y(II) than for PSII quantum yield F_V/F_M (Fig. 2 A). To explain the difference, we first analyzed the impact of HL treatment on non-photochemical dissipation of excitation energy (Fig. 6 B).

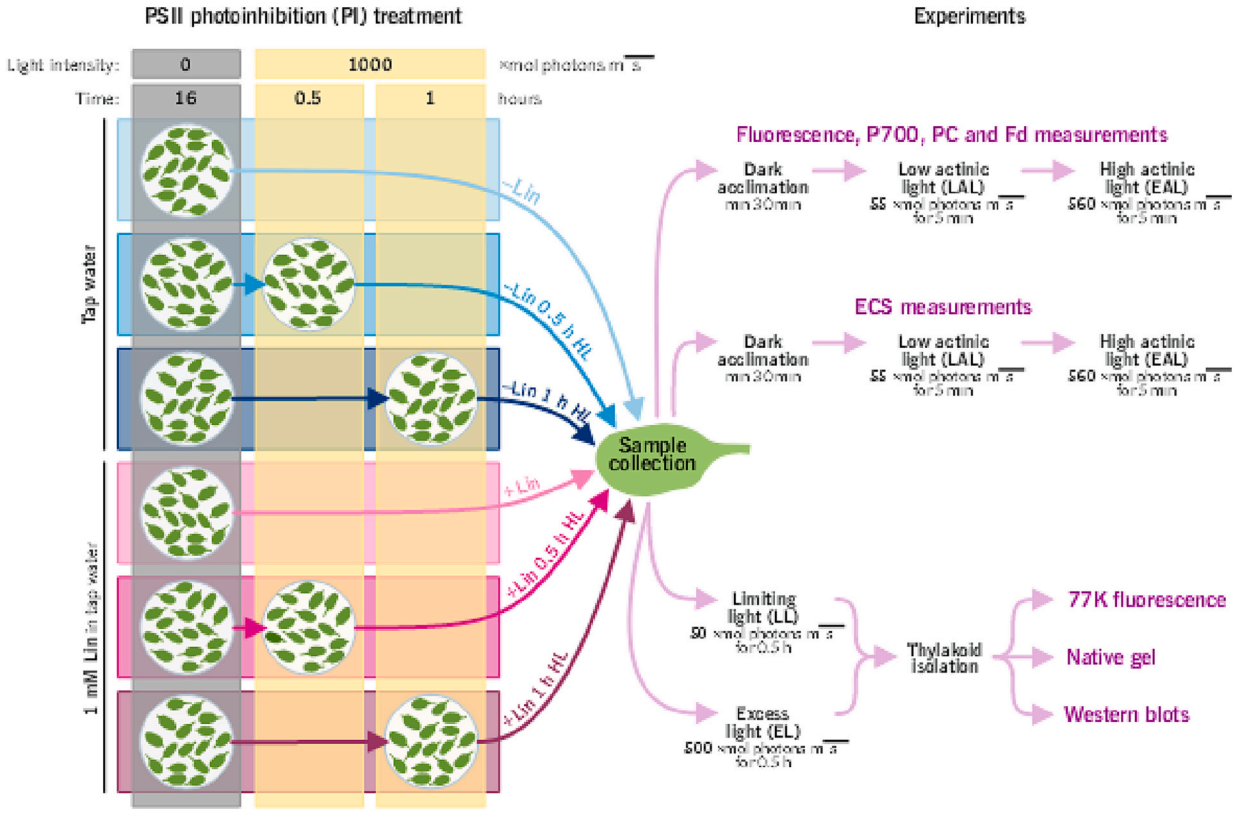

Fig. 1. Experimental setup to induce PSII photoinhibition and to study the consequences of photoinhibition on the function of photosynthetic machinery in limiting and excess light conditions. *Arabidopsis thaliana* wild type was grown for 5–6 weeks under short day growth light of 120 µmol photons m^{-2} s^{-1}. Leaves from several rosettes were detached and incubated in water or 1 mM lincomycin (Lin) on petri plates in darkness for 16 h. Thereafter, the leaves were shifted to high light (HL, 1000 µmol photons m^{-2} s^{-1}) for 0.5 h and 1 h to induce PSII photoinhibition. Leaf samples from the control and photoinhibitory conditions were collected for the fluorescence, P700, plastocyanin (PC) and ferredoxin (Fd) measurements as well as for ECS measurements. Measurements were conducted in limiting (LAL) and excess (EAL) actinic lights after minimum of 30 min dark incubation. Part of the leaves were further treated with low (LL) and excess (EL) lights and then subjected to thylakoid isolation. Thylakoids were used for 77 K fluorescence measurements, western blots and blue native gel electrophoresis.

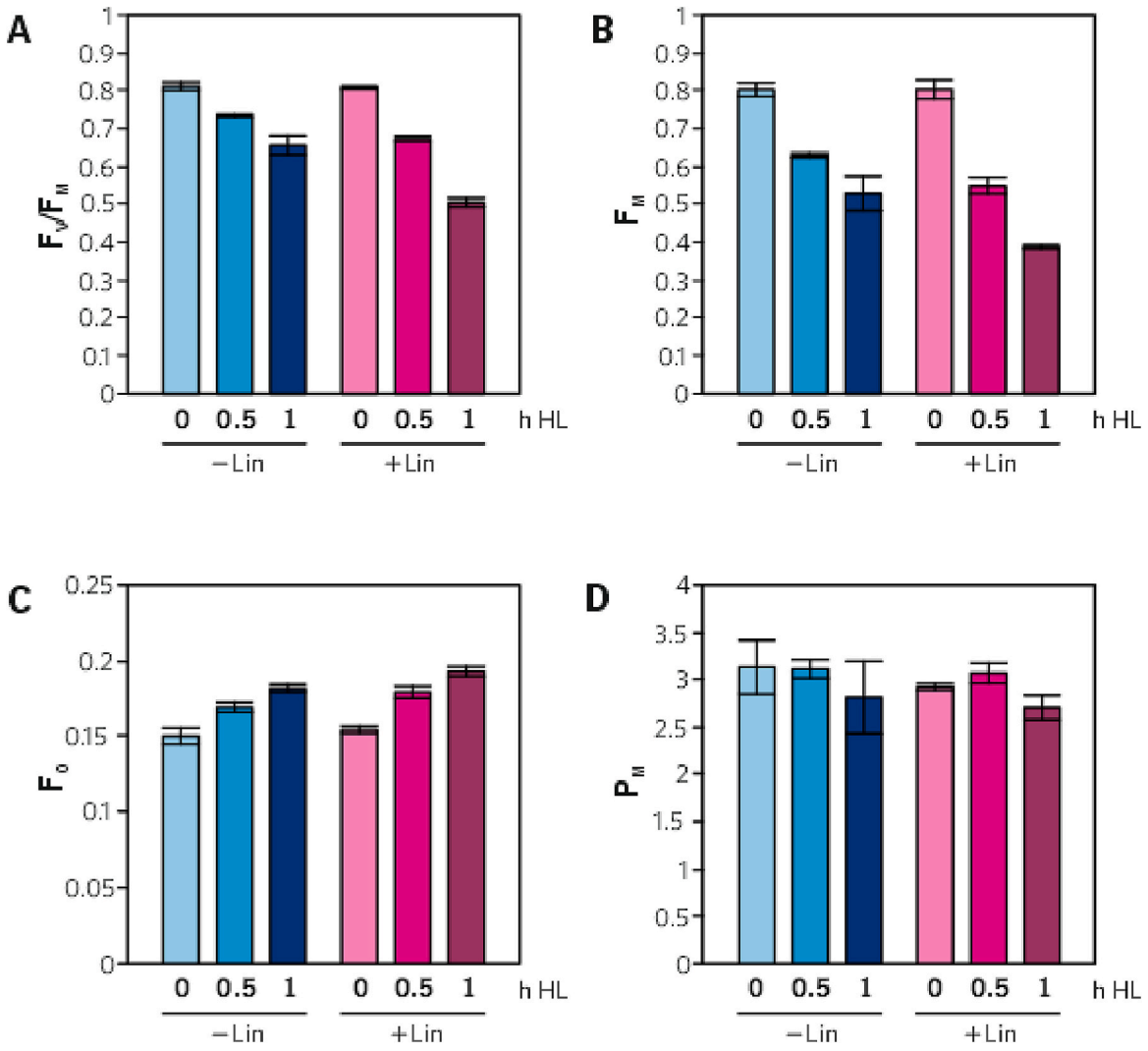

Fig. 2. Effect of 0.5 and 1 h high light treatment (HL) on A. maximal quantum yield of PSII ($F_V/F_M = (F_M–F_0)/F_M$), B. maximal (F_M) and C. minimal (F_0) fluorescence and D. maximal P700 oxidation (P_M). The parameters were measured from leaves incubated overnight in water or in 1 mM lincomycin (Lin) and thereafter high light illuminated (HL, 1000 μmol photons m^{-2} s^{-1}) for 0.5 or 1 h. Error bars show standard deviations among replicates ($n \geq 3$).

In addition to typical actinic light induced NPQ representing the energy-dependent quenching qE regulated by the pH of thylakoid lumen (colored bars), we calculated a novel NPQ value for the photoinhibitory quenching (qI) by using the average F_M values of control and HL treated leaves (gray bars). In Fig. 6 B, the new qI parameter is presented together with the traditionally calculated actinic light-induced NPQ parameter qE. In the absence of Lin, qI increased to about 0.3 in 0.5 h and to 0.5 in 1 h HL treatment and in presence of Lin to 0.5 and 1.0, respectively (gray bars in Fig. 6 B). In EAL, qE was about 1.7 for the control -Lin samples and 1.5 for control +Lin leaves. HL illumination for 0.5 h and 1 h decreased qE for -Lin treated leaves to about 1.2 and 1.1 and for +Lin leaves to 1.0 and 0.5, respectively. Under LAL, qE remained very low, and only minor differences were detectable between the control and photoinhibited leaves (Fig. 6 B). These results are in line with those in [34], which first demonstrated the impact of PSII photoinhibition on actinic light-induced NPQ. Nevertheless, the minor decrease in qE under LAL was not enough to explain, as such, the high Y(II) in relation to F_V/F_M. To get better insights into the properties of the functional PSII centers, we next analyzed the qL parameter that estimates the level of photochemical quenching i.e. the fraction of open PSII RCs when the RCs are assumed to exist in an energetically connected pigment 'lake' [35]. As seen in Fig. 6C, the qL parameter strongly increased after photoinhibitory treatments, with and without Lin, indicating that after PSII photoinhibition the PQ pool remains more oxidized, enabling to keep a larger fraction of PSII centers open. Taking together, PSII photoinhibition decreases the maximal quantum yield of PSII (F_V/F_M), which in turn leads to a decrease in NPQ and electron backpressure towards PSII, resulting actually in smaller impact of photoinhbition on the performance of PSII than estimated from F_V/F_M.

2.3. Impact of PSII photoinhibition on the function of PSI

To evaluate the impact of PSII photoinhibition on electron transfer to PSI, parameters describing the redox state of PSI donor and acceptor sides were measured. Oxidation state of PC under LAL turned out to be very sensitive to changes in the amount of photoactive PSII centers (Fig. 7 A). In LAL, the oxidation level of PC reflected the degree of PSII inhibition. In EAL, all samples remained oxidized due to the photosynthetic control and in the most severely photoinhibited leaf samples probably due to the combined effect of PSII limitation and photosynthetic control. In line with this, the Y(ND), describing the donor side limitation of PSI (i.e. the availability of reduced PC) (Fig. 7 C), increased, while the Y(NA) (Fig. 7 B) parameter, estimating PSI acceptor side limitation (i.e. the availability of oxidized Fd for the incoming electrons), decreased. This suggests that the loss of active PSII and the consequent reduced electron flow from PSII, keep PSI and ETC oxidized

and that the electrons in ETC are unable to saturate the capacity of PSI acceptors even upon a saturating pulse. PSII photoinhibition did not affect the reduction state of Fd under LAL despite changes in other parameters, suggesting that PSII inhibition has the strongest effect on linear electron transfer components in low light.

2.4. PSII photoinhibition decreases the generation of proton motive force, pmf

The impact of PSII photoinhibition on generation of the proton motive force, essential for photosynthetic production of ATP, was assessed next. Pmf is dependent, from one hand, on the rate of electron transfer and, on the other hand, on translocation of protons from thylakoid lumen to the stroma via ATP synthase. Moreover, pmf feedback regulates the electron transfer through Cyt b_6f and extent of NPQ in the light harvesting antenna. In the absence of Lin, HL treatment did not affect the capacity to generate pmf either in LAL or in EAL. In the presence of Lin, HL treatment and the consequent PSII photoinhibition, however, clearly reduced the formation of pmf both in LAL and EAL (Fig. 8).

3. Discussion

Continuous damage and repair of PSII, in all light conditions [10], means that a certain fraction of PSII is always inhibited in light and undergoing different steps of the repair cycle. In high light, when the rate of damage exceeds the rate of repair, inactive PSII centers start accumulating and photoinhibition is manifested at the leaf level [18]. Photoinhibited PSIIs are not able to perform photochemistry and thus the harnessed light energy must either be dissipated non-photochemically in inhibited PSIIs or directed further to a surrounding functional PSII or to another quenching center. The molecular mechanism of photoinhibition-related energy dissipation and the associated fluorescence quenching have remained elusive. PSII photoinhibition is, however, typically measured as quenching of chlorophyll fluorescence that does not relax during the dark period preceding the F_V/F_M measurement (typically 15–30 min) or during the low light phase of NPQ measurement (qI) (typically 5–15 min).

Depending on the point of view, PSII photoinhibition and the following repair cycle can be considered as a harmful and inevitable damage followed by a repair of the damage. PSII photoinhibition and the associated dissipation of excess excitation energy can also be seen as a mechanism providing long-term protection to the photosynthetic machinery [34]. It has been shown, at least in experimental conditions, that PSII photoinhibition can protect PSI from irreversible photodamage [37]. Despite the possible protective role of PSII photoinhibition, it is evident that the decrease in the number of active PSIIs starts at some point limiting the photosynthetic electron flow for optimal NADPH and ATP production. This is the case particularly when the light intensity suddenly decreases, for example due to a cloudy sky, and the situation is overcome only after the damaged PSII centers have been repaired. Here, we investigated the possible impacts of accumulation of photoinhibited PSIIs (Fig. 2) on re-direction of excitation energy to remaining functional PSIIs as well as on the functionality of the entire LET (Figs. 3 - 8) in limiting and excess light. The aim was to disentangle whether the regulatory mechanisms that allow plants to acclimate to changing light conditions are, at the same time, functioning as essential mechanisms to mitigate and acclimate plants to the PSII photoinhibition state as such.

3.1. Photoinhibition increases the relative antenna size of active PSIIs

Photoinhibited PSII centers are not capable of photochemistry, thus reducing the number of PSIIs to be closed by light and emitting fluorescence. Yet, the pigments capturing light energy for the photosynthetic machinery generally remain unaffected by photoinhibition of the reaction center (RC) [38]. Directly after photoinhibition, the damaged PSII RC might function as the quenching center [39]. Nevertheless, when the repair cycle proceeds to a disassembly of the damaged PSII sc, it is highly likely that the capacity for RC quenching is also lost. Thus, the LHC proteins detached from photoinhibited PSII during the repair cycle must either dissipate the energy by themselves or the energy is redirected to the surrounding still active PSIIs that can use the energy for photochemistry or dissipate it by the RC quenching [40]. It is likewise possible that PSII photoinhibition and associated opening of the protein complex structure leads to increased energy transfer to PSI [41]. Nevertheless, independently of the mechanism, the photoinhibition-induced changes in energy transfer result in quenching of chl fluorescence.

In the absence of Lin, 1 h HL treatment induced about 20 % decrease in F_V/F_M (Fig. 2 A) indicating that about one fifth of PSII centers were

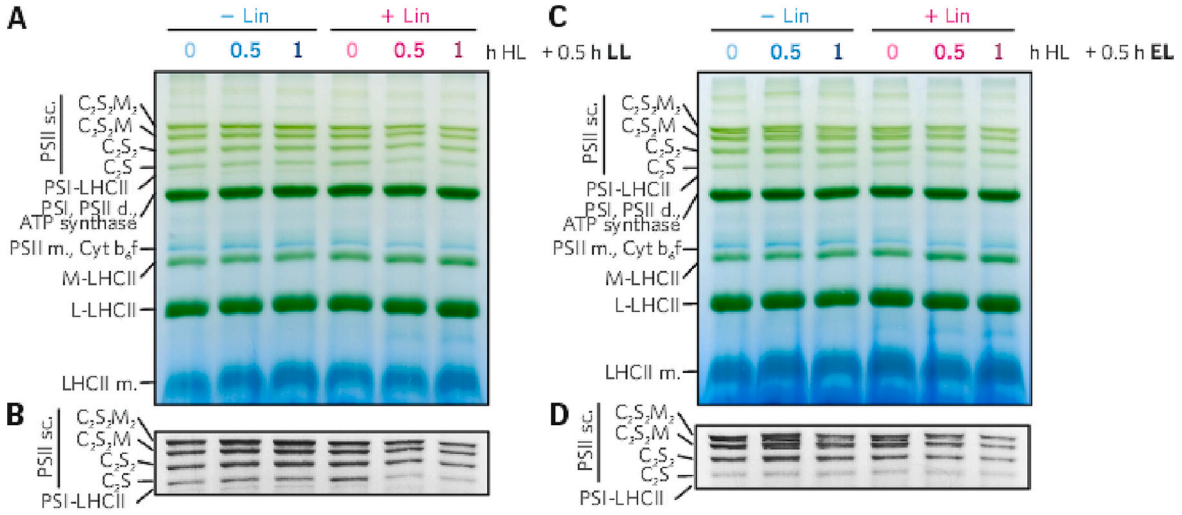

Fig. 3. Effect of 0.5 and 1 h high light treatment on maintenance of thylakoid protein supercomplexes at low and excess light. Thylakoid membranes were isolated from leaves exposed to high light (HL, 1000 μmol photons m^{-2} s^{-1}) for 0, 0.5 and 1 h and then transferred ether to **A.** low (LL, 50 μmol photons m^{-2} s^{-1}) or **C.** excess light (EL, 500 μmol photons m^{-2} s^{-1}) in the absence (-Lin) and presence (+Lin) of Lincomycin (1 mM). **B.** and **D.** contrast enhanced gray scale pictures representing the PSII-LHCII supercomplex (PSII sc) section of the gel to make it easier to see the changes in the complexes. Isolated thylakoids were solubilized by beta dodecyl maltoside and protein complexes were separated by blue native gel electrophoresis. Localization of thylakoid protein complexes are marked on the left side of the BN-gels.

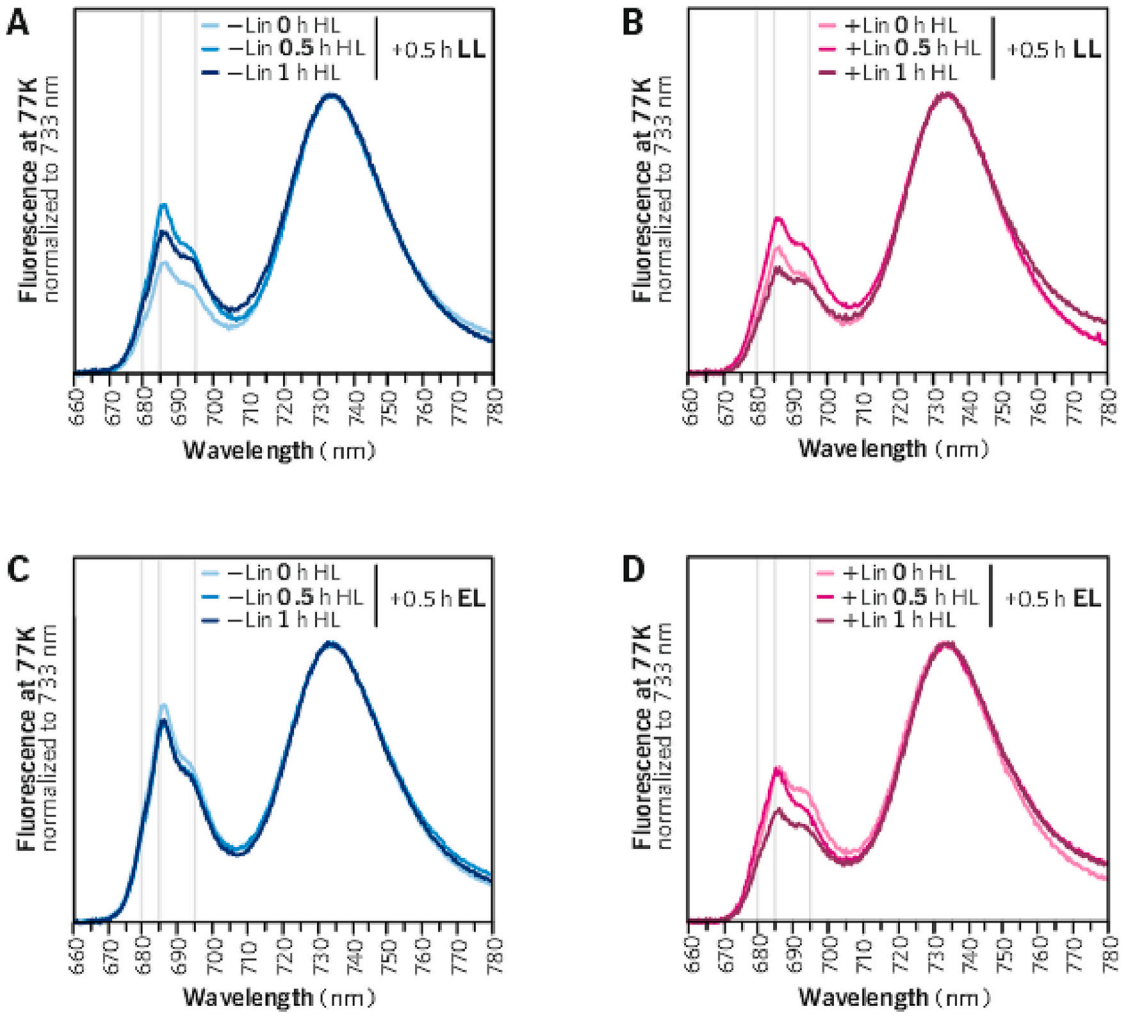

Fig. 4. Effect of 0.5 and 1 h high light treatment on the distribution of excitation energy between PSII and PSI. Fluorescence spectra at 77 K were measured from thylakoid membranes isolated from leaves exposed to high light (HL, 1000 μmol photons m^{-2} s^{-1}) for 0, 0.5 and 1 h and then either to **A, B** low (LL, 50 μmol photons m^{-2} s^{-1}) or **C, D** excess (EL, 500 μmol photons m^{-2} s^{-1}) light for 0.5 h in the absence (-Lin) **A, C** and in the presence (+Lin) **B, D** of Lincomycin (1 mM). Excitation light of 480 nm was used, and the obtained spectra were normalized to 732 nm. The fluorescence peaks 680, 685 and 695 nm are indicated with vertical dotted lines.

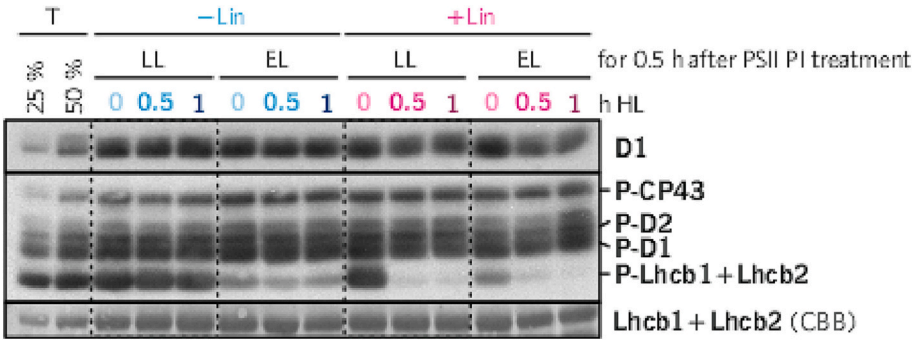

Fig. 5. Effect of PSII photoinhibition on the amount of D1 protein and thylakoid protein phosphorylation. The amount of D1 protein and the intensity of phosphorylated threonine (P-Thr) residues of CP43, D2, D1, Lhcb1 and Lhcb2 proteins were detected with specific antibodies [31] from thylakoids. Thylakoids were isolated from leaves exposed to high light (HL, 1000 μmol photons m^{-2} s^{-1}) for 0, 0.5 and 1 h HL and then either low (LL, 50 μmol photons m^{-2} s^{-1}) or excess (EL, 500 μmol photons m^{-2} s^{-1}) light for 0.5 h in the absence (-Lin) and presence (+Lin) of Lincomycin. Equal sample loading was confirmed by visualizing the total amount of Lhcb1 and Lhcb2 proteins with Coomassie Brilliant Blue (CBB). T = -Lin LL thylakoids dilution series.

inhibited. This was also reflected in the capability of PSII to reduce ETC, especially under LAL, seen as a decrease in the reduction state of PC, decrease in Y(NA) and increase in Y(ND) (Fig. 7). The relative 77 K PSII to PSI fluorescence ratio (Fig. 4) and the integrity of PSII protein complexes (Fig. 3), however, remained unchanged, indicating that the quenching mechanism in response of the decrease in F_V/F_M does not comprise any major changes in thylakoid protein complexes. Under LAL, the lower number of active PSIIs lead to a strong PSII limitation of electron transfer and resulted in oxidation of ETC (Fig. 7), which then inactivated the STN7 kinase and lead to gradual dephosphorylation of LHCII proteins (Fig. 5). Dephosphorylation of LHCII, in turn, increases the relative PSII to PSI excitation (Fig. 4 A), which likely supports the function of still active PSII centers. It is very unlikely that the dephosphorylated LHCII could selectively excite only the functional PSIIs, but instead, could enhance the function of still active PSIIs via PSII connectivity [42]. We therefore propose that the LHCII phosphorylation-

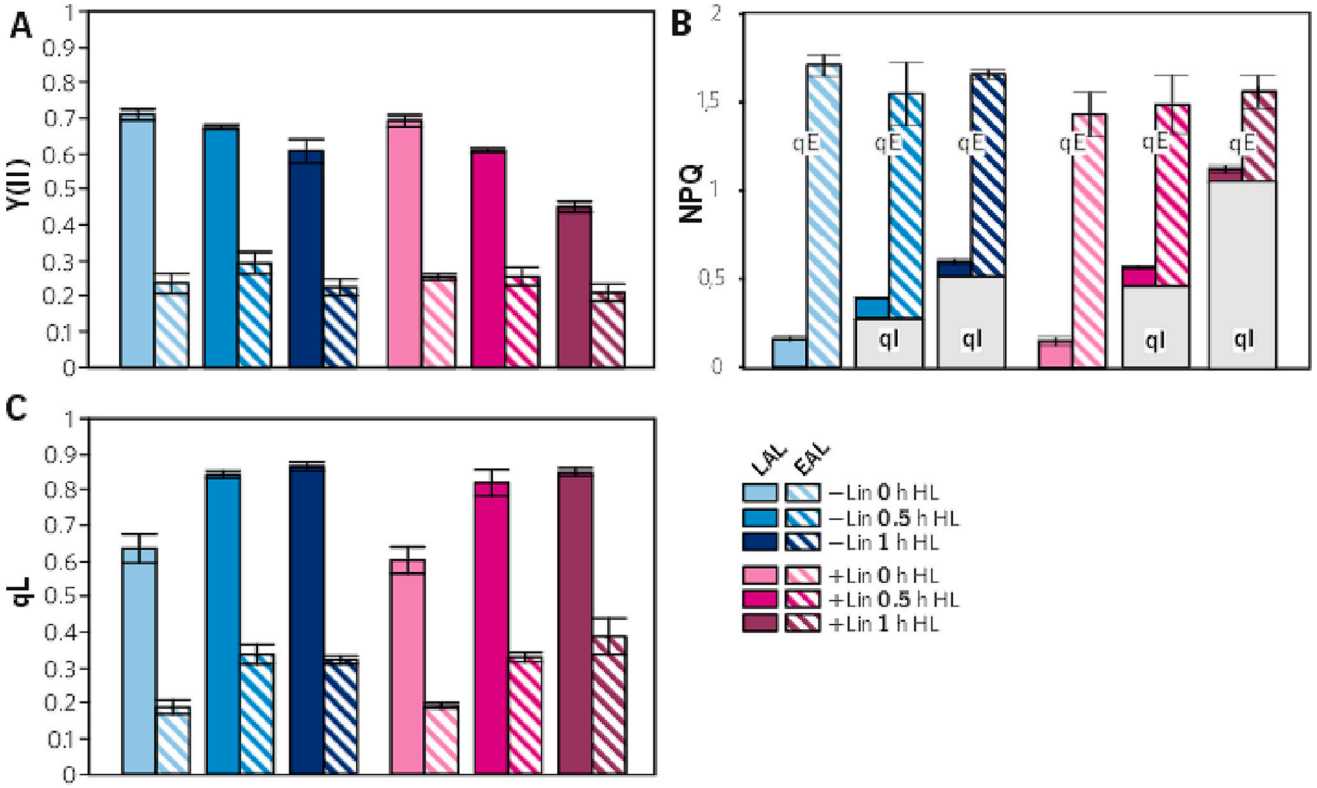

Fig. 6. Effect of PSII photoinhibition on the function of PSII. Leaves exposed to high light (HL, 1000 μmol photons m^{-2} s^{-1}) for 0, 0.5 and 1 h HL and, after 15 min dark incubation, the chlorophyll fluorescence measurements were performed in low (LAL, 55 μmol photons m^{-2} s^{-1}) and excess (EAL, 560 μmol photons m^{-2} s^{-1}) actinic lights (5 mins each). A. Y(II) representing the yield of PSII. B. NPQ colored bars representing the regulated dissipation of excess excitation energy and gray bars representing unregulated PSII-photoinhibition induced quenching qI. C. qL representing the fraction of open PSII centers. Error bars show standard deviations among replicates ($n \geq 3$).

mediated redox regulation of relative PSII to PSI excitation plays an important role in mitigation of the consequences of PSII inhibition, and is implemented by the connectivity between the PSII complexes.

3.2. *Arresting the PSII repair cycle by Lincomycin changes the excitation energy transfer in the thylakoid membrane*

Chloroplast translation inhibitors, Lincomycin (Lin) or Chloramphenicol, are typically used in research of PSII photoinhibition. Such arrest of the repair cycle amplifies the effect of high light stress and enables quick induction of PSII photoinhibition. Similar to the use of any external chemical as an inhibitor, the physiological relevance of using translation inhibitors can be argued. However, the use of translation inhibitor might mimic quite well a long-term CO_2 limitation, heat, low temperature, salt or nutrient stress, which have been shown to lead to an arrest of the PSII repair cycle [36,43]. Here we used Lin, on one hand, with an aim to investigate the impact of strong photoinhibition on the function of photosynthetic light reactions and, on the other hand, to reveal putative differences between the natural and Lin-enhanced photoinhibition of PSII.

With Lin, F_V/F_M decreased by about 20 and 40 % as a response to 0.5 h and 1 h HL treatments, respectively, whilst without Lin, 1 h HL illumination was required for 20 % photoinhibition of PSII centers (Fig. 2 A). Presence of Lin, however, strikingly changed the impact of photoinhibition on PSII protein complexes and the distribution of energy in the thylakoid membrane. In the presence of Lin, photoinhibition led to partial unpacking of PSII sc (Fig. 3), gradual degradation of D1 protein (Fig. 5) and, after 1 h HL, also to a decrease in relative PSII to PSI fluorescence at 77 K (Figs. 4 B and D). When leaves treated at HL for 0.5 h in the presence of Lin were compared with leaves treated at HL for 1 h without Lin (both resulting in 20 % PSII photoinhibition), it turned out that the impact of PSII photoinhibition on LHCII protein dephosphorylation was much stronger in the presence of Lin. Such strong difference in the phosphorylation is hard to explain by a small difference in the redox state of the PQ pool (Fig. 6 C). It is more likely that the unpacking of PSII complexes in grana and migration to grana margins for repair [14] disturb the interaction between the PQ pool, STN7 kinase and the Cyt b_6f complex, and the strong difference in LHCII phosphorylation results rather from structural changes and re-organisations in thylakoid protein complexes than from direct redox regulation of the STN7 kinase. It is possible, as well, that LHCII phosphorylation reflects the redox state of entire PQ-pool, but the qL only the part of the PQ-pool associated with PSII centers that are still active and able to emit fluorescence. Although, in the absence of Lin, HL treatment decreases the quantum yield of PSII (Fig. 2 A) indicating accumulation of damaged PSII centers, the thylakoid protein complexes are mostly intact due to the efficient repair machinery. In these “natural” conditions, the damaged D1 protein is replaced in a very controlled manner without long-term loss in the capability for RC quenching in damaged or nearby PSII centers. On the contrary, when the synthesis of D1 protein is arrested by Lin, the incomplete PSII repair leads to an unpacking of PSII sc. It is impossible to argue whether this is an experimental artefact caused by Lin or whether it is analogous to the unpacking of PSII sc seen in conifers upon sustained winter acclimation [44]. Interestingly, after 0.5 h HL with Lin, the dephoshorylation of LHCII was accompanied by enhanced relative excitation of PSII (Fig. 4B), but when the PSII inhibition progressed further and PSII sc became partially disassembled (Fig. 3), the PSII to PSI 77 K fluorescence emission ratio decreased (Fig. 4 D). Taking these results together, the disassembly of PSII sc appears to prevent the enhanced excitation of still functional PSIIs by dephosphorylated LHCII complexes, as observed in the absence of Lin. It is conceivable that the PSII sc are principally a prerequisite for segregation of PSII and PSI, but

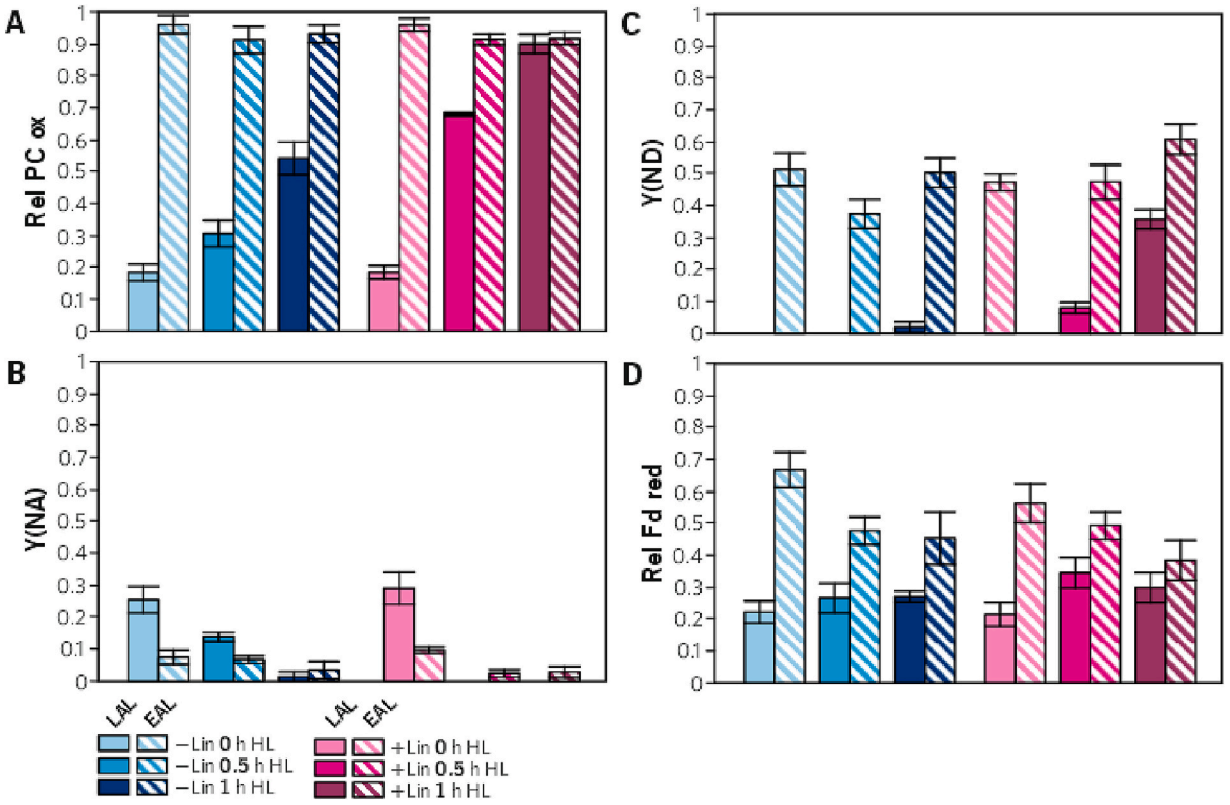

Fig. 7. Effect of PSII photoinhibition on electron transfer to and from PSI. Leaves were treated and measurements were conducted as explained in Fig. 6. A. Oxidation level of PC (Rel PC_{ox}), B. acceptor side limitation of PSI Y(NA), C. donor side limitation of PSI Y(ND) and D. reduction state of ferredoxin (Rel Fd red). Error bars show standard deviations among replicates ($n \geq 3$).

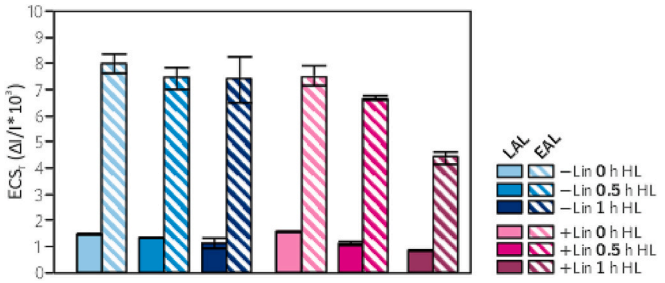

Fig. 8. Effect of PSII photoinhibition on generation of the proton motive force under low light and excess light illumination. ECS_t was measured with the dark interval relaxation kinetics method after a minimum of 30 min dark acclimation. Error bars show standard deviations among replicates (n ≥ 3).

such a strict lateral segregation is lost when the PSII sc complexes disassemble in grana margins. This also results in collection of light energy by smaller PSII-LHCII complexes, which have a better access to PSI that is a strong quencher of excitation energy. Nevertheless, PSII inhibition reduces the number of active PSII centers and the excitation of PSII further decreases upon the loss of thylakoid lateral heterogeneity, thus limiting the electron transfer to PSI and ensuring that PSI can safely quench the excess excitation energy.

The presence of Lin clearly changed the impact of PSII photoinhibition on remaining functional PSII complexes as well as on the energy distribution in the thylakoid membrane but, additionally, it revealed that the photoinhibition-related quenching mechanisms are different depending on whether the PSII repair machinery is functional or not. As long as the accumulation of PSII sc in the thylakoid membrane is strictly controlled (-Lin), it is likely that the photoinhibitory quenching (qI) occurs in PSII complexes at physiological temperatures. On the contrary, when the repair cycle is arrested (+Lin), the PSII sc complexes disassemble and the relative PSII to PSI fluorescence at 77 K decreases, suggesting that quenching is related to structural changes in the thylakoid membrane, most likely to partial loss of lateral heterogeneity and consequent spillover of excitation energy to PSI. If similar series of reactions take place under natural conditions, for example upon a low temperature stress, it would be capable of providing efficient long-term quenching that can protect the photosynthetic machinery from further damage.

3.3. *Impact of PSII photoinhibition on functionality of photosynthetic light reactions*

Maximizing the efficiency of photosynthesis under PSII photoinhibition conditions, plants need to respond to PSII limitation by tuning the regulation of excitation energy distribution and electron transfer to make use of the remaining active PSIIs in the best possible way. Photoinhibition decreases the number of PSII that can be closed by a saturating pulse and induce maximal fluorescence emission, evidenced as a decrease in F_M (Fig. 2 A). Photoinhibition also decreases the fluorescence induced by actinic light (F′) (Supplemental Fig. S1). Indeed, the photoinhibited PSII centers lack the kinetics of variable fluorescence and thus, the fluorescence signals used to calculate the parameters originate exclusively from uninhibited PSII centers. As shown in Fig. 6 C, the NPQ induced by EAL (4 x GL intensity) is inversely proportional to the level of PSII photoinhibition, indicating that the less active the PSII centers the lower is the NPQ (Fig. 6 B), reported also earlier in [45]. Therefore, the light harvesting efficiency of LHC systems remains high despite PSII photoinhibition, enabling enhanced light harvesting of still active PSII centers and Y(II) remains high in EAL (Fig. 6 A). At the same time, in line with earlier results [46] the fraction of open PSII reaction centers (qL) increases, suggesting that the backpressure towards PSII from the PQ

pool decreases as the result of photoinhibition. Decrease in NPQ together with increased qL provide evidence that the excitation turnover of the still active PSIIs, after the PSII inhibition treatment, is faster than that of average PSIIs before the inhibition treatment in the same light intensity. Indeed, 20 % photoinhibition does not increase P700 oxidation Y(ND) and 40 % photoinhibition increases it only slightly in EAL conditions (Fig. 7 C). Our results provide evidence that when the light energy is not limiting, even strong PSII limitation can be compensated by enhancing the energy collection capacity of functional PSIIs and thereby also the rate of linear electron flow. The 40 % PSII inhibition was induced in the presence of Lin and, as described above, it is likely that such Lin-enhanced inhibition leads to energy spillover from PSII to PSI due to putative unnatural disassembly of PSII complexes. It is conceivable that in natural conditions (− Lin), the maintenance of PSII sc integrity by strictly controlled PSII repair cycle along with dephosphorylation of LHCII proteins at high light, provide conditions that can substantially enhance the excitation of non-inhibited PSII, enabling plants to cope with even stronger photoinhibition. The level of NPQ and the resistance against electron flow at Cyt b_6f are regulated by the proton concentration of the thylakoid lumen. PSII inhibition decreases pmf at EAL (Fig. 8), indicating a decrease in the proton concentration of thylakoid lumen and accordingly lower NPQ and less photosynthetic control at Cyt b_6f. This is in line with the finding that about the same 50–60 % of P700 is oxidized (Y(ND)) (Fig. 7) independently of the amount of active PSII centers (Fig. 2A). In LAL light (40 % of GL), the decrease in F_V/F_M is reflected to the oxidation of P700 and PC more straightly indicating that in low light the proper number of active PSIIs are needed to maintain sufficient electron transfer to PSI.

3.4. Summary

PSII photoihibition leads to increased oxidation of PQ-pool in low light and decreased generation of the proton motive force (pmf) at high light. These changes have impact on both the electron transfer and distribution of excitation energy. Independently of the severity of PSII photoinhibition, the decrease in the number of active PSII leads to oxidation of PQ-pool that decreases the backpressure towards electron transfer, thus facilitating the function of remaining PSII. The decrease in pmf, on the contrary, downregulates the photosynthetic control at Cyt b_6f, enhancing the electron transfer especially in high light. Opposite to electron transfer, the impact of photoinhibition on distribution of excitation energy is dependent on the severity of PSII photoinhibition. When only a small fraction of PSII centers is inhibited, the structure of PSII sc remains intact. In these conditions, lateral heterogeneity of the thylakoid membrane and the regulation of excitation energy distribution between PSII and PSI by LHCII phosphorylation remain functional. It is also likely that the site of photoinhibitory quenching resides inside the intact PSII sc. When the repair cycle of PSII is inhibited, PSII sc become gradually disassembled, leading to a loss of structural integrity maintaining normal lateral segregation between PSII and PSI and enabling LHCII phosphorylation-based regulation of excitation energy distribution. It is also highly likely that when PSII sc become disassembled, the inhibited PSII loses the capacity for excitation energy dissipation but due to the concomitant loss of lateral segregation of thylakoid membrane, PSII RC quenching is replaced by excitation energy spillover to PSI and quenching by PSI. Importantly, the fact that PSII inhibitory illumination enhances the function of non-inhibited PSII centers, indicates that the Fv/fm parameter overestimates the consequences of PSII photoinhibition on the overall rate of PSII electron transfer.

4. Materials and methods

4.1. Plant material and their growth and treatment

Wild type *Arabidopsis thaliana* (ecotype Colombia) was grown at 23 °C with short day regime: 120 μmol photons m^{-2} s^{-1} light for 8 h and dark for 16 h (growth light, GL) with OSRAM PowerStar HQIT 400/D metal halide lamps as the light source. For the experiments, fully expanded 5–6-week-old leaves were detached and incubated in 1 mM Lin on petri plates in darkness overnight (16 h) (Fig. 1). After the darkness, the leaves were subjected to high light (HL, 1000 μmol photons m^{-2} s^{-1}) with Heliospectra L4A for 0.5 and 1 h. Leaf samples for DUAL-KLAS-NIR and DUAL-PAM measurements were collected directly after each light treatment (Lin, Lin + 0.5 h HL and Lin + 1 h HL). Before thylakoid isolation, leaves were additionally treated with either low light (LL, 50 μmol photons m^{-2} s^{-1}) or excess light (EL, 500 μmol photons m^{-2} s^{-1}) with OSRAM PowerStar HQIT 400/D metal halide lamps for 30 min.

4.2. Thylakoid isolation and chl determination

Thylakoids were isolated after the overnight Lin incubation as well as after the subsequent light treatments (Fig. 1). First, the leaves were grinded in 50 mM Hepes/KOH pH 7.5 supplemented with 330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 5 mM ascorbate, 0.05 % BSA and 10 mM NaF, after which the homogenate was filtered through Miracloth and the chloroplasts were collected by centrifugation at 2500 ×*g* for 5 min at 4 °C. Thylakoids were released with 50 mM Hepes/KOH pH 7.5 containing 5 mM sorbitol and 10 mM NaF, collected by centrifugation at 2500 ×*g* for 5 min at 4 °C. The thylakoids were washed with storage buffer (50 mM Hepes/KOH pH 7.5 containing 100 mM sorbitol, 10 mM $MgCl_2$ and 10 mM NaF) and centrifugation at 2500 ×*g* for 5 min at 4 °C and finally stored in the same buffer. Chl concentration was determined according to [47] from 1 μl of thylakoids in storage buffer diluted into 1000 μl of 80 % acetone buffered with 2.5 mM Hepes/KOH pH 7.5.

4.3. Fluorescence, P700, PC and Fd measurements

Photochemistry of PSII and PSI and steady state redox states of PC and Fd were measured from Lin, Lin + 0.5 h HL and Lin + 1 h HL-treated and control leaves with DUAL-KLAS-NIR (Heinz Walz GmbH). Leaves were illuminated with 55 μmol photons m^{-2} s^{-1} 635 nm red actinic light for 5 min after which light intensity was increased to 560 μmol photons m^{-2} s^{-1} for 5 min. Saturating pulses (8000 μmol photons m^{-2} s^{-1}) of 800 ms were given at the end of both light intensities. Chl *a* fluorescence was detected with 460-nm measuring light (1 μmol photons m^{-2} s^{-1}), while P700, PC and Fd redox states were determined by deconvolution of dual-wavelength 785–840, 810–870, 870–970, and 795–970 nm signals [48]. Prior to the measurements, the photosynthetic machinery was dark-acclimated for minimum of 30 min (Fig. 1). After dark-acclimation, minimal (F_0) and maximal fluorescence (F_M) were measured. F_0 value under actinic light was estimated as $F_0' = F_0/[(F_V/F_M) + (F_0/F_{M'})]$ [49], while $F_{M'}$ is directly measured. Similarly, maximal P700 oxidation (P_M), maximal PC oxidation (PC_M) and maximal Fd reduction (Fd_M) signals were measured after the dark-acclimation with NIR Max-script [50]. While steady state P700 oxidation (P), PC oxidation (PC_{ox}) and Fd reduction (Fd_{red}) were determined under actinic light. From these variables, maximal quantum yield of PSII was estimated with $F_V/F_M = (F_M–F_0)/F_M$ [51], photochemical quenching of PSII fluorescence compatible on lake model i.e. fraction of open PSII RCs with $qL = (F_M'–F)/(F_M'–F_0') \times (F_0'/F)$ [52], energy-dependent non-photochemical quenching with NPQ = (F_M / F_M ')-1 and photoinhibatory NPQ (qI) with (average F_M of control samples/average F_M of HL treated samples)-1. Donor side limitation i.e. oxidation state of PSI with $Y(ND) = (P - P_0)/(P_M - P_0)$ and acceptor side limitation of PSI with $Y(NA) = (P_M–P_{M'})/P_M$ [53,54]. Steady state oxidation of PC was calculated as Rel PC ox = PC_{ox}/PC_M and steady state reduction of Fd was calculated as Rel Fd red = Fd_{red}/Fd_M.

4.4. Electrochromic shift measurements

Electrochromic shift was measured with DUAL-PAM-100 equipped

with P515/535 module (Heinz Walz GmbH). Leaves were illuminated with 55 μmol photons m^{-2} s^{-1} 635 nm red actinic light for 5 min after which light intensity was increased to 560 μmol photons m^{-2} s^{-1} for 5 min. 250 ms dark interval was given at the end of both light intensities to quantify thylakoid proton motive force (ECS_T). Electrochromic shift was determined by the difference of 515 nm and 550 nm signals [55]. ECS_T was calculated as the difference between the ECS in the light before the dark interval and the ECS dark baseline at the end of dark interval.

4.5. Fluorescence measurements at 77 K

Fluorescence emission spectra of isolated thylakoids were recorded at 77 K (liquid nitrogen) by using an Ocean Optics QE Pro Spectrometer. The thylakoids were diluted with storage buffer (50 mM Hepes/KOH pH 7.5 containing 100 mM sorbitol, 10 mM $MgCl_2$ and 10 mM NaF) into a final concentration of and 2 μg chl / 200 μl and excited at 480 nm. The spectra were normalized to 732 nm, the highest data point of the control sample.

4.6. Western blotting and blue native gel electrophoresis

For immunoblots, thylakoid membrane proteins were solubilized and denatured with a solution containing 138 mM Tris/HCl pH 6.8, 6 M urea, 22.2 % (*v*/v) glycerol, 4.3 % (*w/v*) SDS and 10 % (v/v) β-mercaptoethanol and separated from the insoluble material by centrifugation at 3952 ×*g* for 5 min at room temperature. The proteins of thylakoid membranes of were separated with SDS polyacrylamide gel electrophoresis (SDS-PAGE) with 15 % acrylamide and 6 M urea and transferred onto PVDF membrane (Millipore). The remaining binding surface of the membrane was blocked with 5 % (w/v) BSA (Sigma-Aldrich). The D1 protein was recognized with an antibody from Research Genetics [56] and phosphorylated threonine residues with an antibody from New England Biolabs (catalogue number 6949S). The antibody signals were visualized with horseradish peroxidase-linked secondary antibody (Agrisera) and Amersham ECL Western blotting detection reagents (GE Healthcare). To ensure equal sample loading, all proteins were visualized with 0.1 % Coomassie Brilliant Blue in 40 % (v/v) methanol and 10 % (v/v) acetic acid.

4.7. Native gel electrophoresis

Isolated thylakoids (5 μg chl per treatment) were solubilized with beta dodecyl maltoside and solubilized complexes were separated with blue native gel electrophoresis as described in [57].

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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