Photosystem I functions in non-photochemical energy dissipation when its iron-sulphur clusters are photodamaged

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Abstract

Photosystem I (PSI) uses light energy and electrons from photosystem II (PSII) to produce NADPH. PSI has two faces; on one hand it is very tolerant against excess light, whilst on the other hand extremely sensitive to excess electrons from PSII (*i.e.* when the utilisation of electrons is blocked on reducing side of PSI). It has been assumed that PSI is well protected from photoinhibition due to strict control of intersystem electron transfer chain (ETC). Here, it is demonstrated that the Iron-Sulphur (FeS)-clusters of PSI are more sensitive to high light stress than previously anticipated, yet the PSI with damaged FeS-clusters still functions as non-photochemical photoprotective energy quencher (PSI-NPQ). Upon photoinhibition of PSI, the highly reduced ETC further triggers thylakoid phosphorylation-based mechanisms that increase energy flow towards PSI. It is concluded that the sensitivity of FeS-clusters provides an additional photoprotective mechanism that is able to down-regulate also PSII, based on PSI quenching and protein-phosphorylation.

Introduction

Photosystem I (PSI) is very tolerant against excess energy and has been reported to become damaged only under very specific stress conditions when the transfer of electrons to PSI exceeds the capacity of the PSI electron acceptors[1-4]. PSI photoinhibition was initially described as an oxidative destruction of the iron-sulphur (FeS)-clusters of PSI in chilling sensitive cucumber plants due to inactivation of superoxide dismutase[3]. More recently, evidence has accumulated indicating that PSI is more susceptible to damage than PSII under fluctuating light conditions[5, 6]. Depending on the phylogenetic position inside the green lineage, oxygenic photosynthetic organisms have evolved with different mechanisms to protect PSI against photodamage, either by limiting electron transfer to PSI or by providing an alternative limitless sink for the electrons[7].

In plants, PGR5 and PGRL1 proteins-dependent mechanism is particularly important for PSI photoprotection under relatively short exposure of plants to high light (HL)-stress, before the PSII activity becomes down-regulated via photoinhibition[5, 6, 8-10]. The absence of PGR5 has been shown to cause defective formation of transthylakoid proton gradient (ΔpH), lack of induction of non-photochemical quenching (NPQ), lack of P700 oxidation[11, 12], sensitivity to low CO_2 conditions[13] and severe damage to PSI upon shift of plants to HL[5, 6, 10, 12, 14].

From one hand, PSI is extremely tolerant against excess excitation energy, yet on the other hand, it is very sensitive to excess of electrons from PSII[1, 5]. The physiological importance of these two different facets of PSI has remained elusive. Here, we have addressed the mechanism and physiological role of PSI photoinhibition by

extensive comparative analysis of WT and the *pgr5* mutant plants deficient in strong light-induced ΔpH. Based on our experimental data, it is suggested that the photodamage of PSI is a novel photoprotection mechanism allowing plants to acclimate to varying light and temperature conditions.

Results

Photo-oxidation of PSI in WT and the* pgr5 *mutant – Light-induced oxidation of P700 has been reported to be missing in the *pgr5* mutant[11, 15]. Therefore, an intense far red (FR) light, which preferentially excites PSI, was required to obtain the fully oxidized state of PSI in the *pgr5* mutant (Fig. 1). As demonstrated in Fig. 1a,b, the oxidation of P700 took place upon illumination with FR light, and reached a plateau within 7-9 s, both in WT and *pgr5*. The maximum level of oxidized P700 (denoted as Pm) was induced by a saturating pulse of actinic light in continuous FR light. Further, application of a single turnover (ST) and a multi turnover (MT) pulse of saturating actinic light activates few turnovers of electrons supplied by PSII, therefore transiently reducing $P700^+$, which is subsequently oxidized back due to continuous exposure to FR light. Similar reduction and re-oxidation kinetics of $P700^+$ upon ST (at 35 s of recording) and MT (at 45 s of recording) pulses both in WT and *pgr5* (the lowest traces in Fig. 1a,b) indicated the absence of any distinct alternate electron transfer pathways, specifically dependent on the PGR5 protein in WT. Similarly, P700 oxidation-reduction kinetics remained completely unaffected in WT upon transferring the plants to HL for up to 120 min (Fig. 1a). Conversely, already within 30 min exposure of the *pgr5* leaves to HL, the oxidation of P700 was severely declined to a level less than 30% of that of untreated *pgr5* plants, furthermore, almost no oxidation was observed in 60 and 120 min HL-treated *pgr5* leaves.

Similar experiments were repeated with isolated thylakoids, where linear electron transfer (LET) from PSII to PSI remains functional, but the further electron transfer on reducing side of PSI, from ferredoxin (Fd) via FNR to $NADP^+$ or from Fd to NDH-dependent cyclic electron transfer[12], is mainly absent due to removal of the stromal compartment. To this end, thylakoids were isolated from HL-treated leaves, followed by monitoring of the PSI redox kinetics. Accordingly, when the ST and MT pulses were applied, rapid oxidation and re-reduction of $P700^+$ was observed in thylakoids of both WT and *pgr5*, which was followed by slower re-oxidation (compared to Fig. 1a,b) until a plateau was reached (Fig. 1c,d). The rate of re-oxidation by FR light was also slower both in WT and *pgr5* after application of saturating pulses of actinic light. This indicated the presence of functional water oxidation complex and normal electron supply from the transiently activated PSII by a saturating light pulse. However, low acceptor side electron transfer from PSI caused a delay in reaching the steady state level of P700

oxidation. Thylakoids from HL-treated WT showed exactly similar pattern of oxidation-reduction and re-oxidation responses upon the ST and MT pulses as the non-treated control WT leaves. In sharp contrast, the thylakoids from HL-treated *pgr5* leaves could hardly generate the oxidized state of P700, providing evidence that the lack of electron transfer via FeS-clusters to the acceptor side components prevented charge separation or caused higher back reactions, which completely inhibited the oxidation of P700.

***Susceptibility of PSI and PSII to photoinhibition in WT and the* pgr5 *mutant* –** Room temperature (RT) EPR measurements were performed in the course of the 2h HL-treatment of intact leaves to determine the amounts of functional PSI and PSII in the thylakoid membrane of WT and *pgr5* plants. Two EPR signals can be observed at RT: dark stable radical signal from $TyrD^{\bullet}$ in PSII and light- or chemically-oxidized radical signal from $P700^{+}$ in PSI[16]. Both EPR signals at their maximally induced size correspond to one spin per respective reaction centre and therefore are precise probes for quantification of the functionality of both photosystems[16]. The results of such EPR measurements on $TyrD^{\bullet}$ and $P700^{+}$ from WT and the *pgr5* mutant are shown in Fig. 2a,b (Supplementary Table S1).

After two hours of HL-treatment, the amount of functional PSII centres had decreased similarly both in WT and the *pgr5* mutant to about 70-80% of the initial value (Fig. 2a,c). The amount of functional PSI centres in WT had similarly decreased to about 70% of the initial value after the 2h HL-treatment (Fig. 2b,c). On the contrary, dramatic decrease was observed in the amount of functional PSI in the *pgr5* mutant after 2h HL-treatment of intact leaves, being less than 25% of the pre-HL level (Fig. 2b,c). The PSI/PSII ratio showed pronounced decrease already after 30 min of HL-treatment in the *pgr5* mutant, while in the WT it remained almost unchanged during the entire HL-treatment of two hours (Supplementary Table S1).

The kinetics of light inducible $P700^{+}$ reduction was also affected during the 2h HL-treatment (Fig. 2d,e). In WT it was found to become 2-3 times slower after the HL-treatment, although the amplitude of the light-induced signal was affected much less (Fig. 2d). In contrast, in the *pgr5* mutant the amplitude of the light-induced $P700^{+}$ signal had decreased by 75% already after 30 min of the HL-treatment (Fig. 2e) and also showed much slower kinetic decay than in WT. Thus, the EPR measurements at RT demonstrated that in *pgr5*, both the amount and the electron transfer properties of the PSI complex are highly vulnerable upon HL-treatment of the leaves, to a far more extent than those of PSII. HL-treatment induced PSI damage also in WT but to a far less extent than in the pgr5 mutant (Supplementary Table S1). It is likely that PSII photoinhibition, on the top of the ΔpH-dependent slow-down of electron transfer, is suffient to protect PSI from further damage. On the contrary, in the absense of ΔpH-dependent regulatory mechanisms, the rate of PSII photoinhihibition is not sufficient enough to protect PSI[10].

Damage of PSI FeS components in WT and the* pgr5 *plants – Next, low temperature EPR spectroscopy was applied to investigate the acceptor side of the PSI complex in WT and the *pgr5* mutant. It is possible to induce stable charge separation and to observe the reduction of terminal acceptors in PSI, the FeS-clusters F_A and F_B, by illumination at liquid Helium (He) temperatures (16 K) where both F_A and F_B become visible by EPR in their reduced form. Illumination of PSI at 16 K normally results in a mixture of clusters with either F_A^- or F_B^- present on the acceptor side of PSI[17]. Both of them are easily observable as recognizable EPR signals in light minus dark difference spectra.

EPR spectra from WT and the *pgr5* mutant, obtained after illumination of dark-incubated thylakoid membranes at 16 K, are shown in Fig. 3. In WT thylakoids isolated from dark-incubated leaves, efficient reduction of F_A and/or F_B was observed after 6 min illumination at 16 K. HL-treatment of WT plants for 30 min resulted only in 50% of reduced F_A and F_B clusters as compared to dark-incubated WT leaves and this amount did not change upon continued HL-treatment of plants for two hours (Fig. 3a). In thylakoids isolated from dark-incubated *pgr5* mutants, only half of the F_A/F_B clusters were reduced by illumination at 16 K, as compared to the dark incubated WT plants (Fig. 3b). Exposure of the *pgr5* mutant to HL for 30 min decreased the amount of reduced F_A and F_B clusters even more, by about three times and after two hours of HL, the amount of reduced FeS-clusters in PSI of the *pgr5* mutant had decreased to 25% of the mutant dark control value or to 12% or less if compared to the WT control (Fig. 3b). The fraction of reducible F_A and F_B can be seen by comparing the intensity of the shoulder (F_B cluster) and the peak (F_A cluster) at the g_z region (marked with * in Fig. 3). Despite strong decline of the F_A/F_B EPR signals after HL-treatment of WT and the *pgr*5 mutant, the PsaC and PsaD proteins that function in FeS-cluster binding and stabilizing [18], respectively, did not degrade upon HL illumination of leaves (Fig. 3c). Indeed, the amount of overall PSI complexes was not affected as the content of the PsaA and PsaB subunits also remained unchanged (Fig. 3c).

Fluorescence emission spectra indicate enhanced excitation energy transfer to PSI in* pgr5 *after the damage of the FeS-clusters – In order to investigate the effect of PSI photoinhibition on the fate of excitation energy, the fluorescence emission spectra at 77 K and at room temperature (RT) were recorded (Fig. 4a,b). At RT, PSI is a very efficient quencher of excitation energy. Decreasing the temperature, however, slows-down the migration of excitation energy towards the quenching process, and at 77 K PSI is already a strong fluorescence emitter. Based on this, the comparison of the fluorescence emission spectra obtained at 77 K (quenching mechanism by PSI largely prevented) to that obtained at RT (quenching by PSI efficient) provides information about the intactness of the intra-PSI energy transfer reactions required for the quenching process (Fig. 4c,d). In

WT, a minor increase in relative PSI emission at 77 K was detected in the course of HL-treatment. In contrast to WT, *pgr5* showed a strong increase in relative excitation of PSI (Fig. 4c,d), despite the fact that PSI centres were largely photoinhibited. In line with 77 K emission spectra, a minor increase in the course of HL-treatment of intact leaves was observed also in the relative PSI fluorescence of the RT spectra (Fig. 4a,b), and this increase was much more pronounced in *pgr5* than in WT. This provided strong evidence that the damaged PSI can still function as a thermal dissipator of excitation energy.

In order to get a deeper insight into the effects of PSI photoinhibition on the light harvesting system, the 77 K excitation spectra were recorded from thylakoids isolated from growth light (GL)-acclimated and 2 h HL-treated WT and *pgr5* plants (Fig. S1). As expected, the increased relative excitation of PSI was accompanied by an increase in chl *b* excitation of PSI. Nonetheless, a similar change was observed in the excitation spectra of PSII as well. Thus, the phosphorylation of light harvesting complex II (LHCII) and consequent antenna migration cannot provide a simple solution. Instead, the contribution of elevated chl *b* excitation to both PSI and PSII corroborated our earlier reports[9, 19] and are in line with emerging new vision on regulation of excitation energy distribution to the two photosystems, being based on reorganization of the entire LHCII system and not on migration of only a specific LHCII antenna fraction[20-23].

***Enhanced thylakoid protein phosphorylation in* pgr5 *by HL-treatment* –** Based on results described above, it seemed obvious that the high 77 K PSI fluorescence emission was not a direct consequence of only the photodamage to PSI. Another option is that the increased PSI fluorescence upon HL-treatment is due to increased antenna cross section of PSI. PSI antenna cross section is regulated via reversible phosphorylation of both the PSII core and the LHCII proteins, only the concomitant phosphorylation of both complexes increasing the relative PSI antenna cross section[9]. The STN7 kinase, which phosphorylates the LHCII proteins, is activated by binding of plastoquinol (PQH_2) to the Qo site of the Cyt b_6f complex, whereas the inhibition of LHCII phosphorylation occurs by accumulation of reductants in chloroplast stroma at HL[24]. Contrary to LHCII phosphorylation, the maximal PSII core protein phosphorylation requires strong reduction of the PQ-pool[25, 26] and indeed, in *pgr5* the PSI photodamage stops the LET through PSI and results in accumulation of electrons in the PQ-pool. To investigate the thylakoid phosphorylation pattern in HL-treated *pgr5* leaves, thylakoids were isolated and the phosphorylation was quantified by immunoblotting with the P-Thr antibody. As shown in Fig. 4e, the thylakoid proteins were moderately phosphorylated both in WT and *pgr5* under growth light and as revealed by 77 K fluorescence spectra, there was no difference in the excitation balance between WT and *pgr5* (Fig. 4e). Shift of leaves to HL led to an increase in phosphorylation of PSII core proteins both in WT and *pgr5*. In

WT, the increased PSII core protein phosphorylation was accompanied with dephosphorylation of the LHCII proteins (Fig. 4e) and no remarkable change in the relative PSI excitation was recorded, in line with earlier observations[9, 21, 24]. On the contrary, HL did not induce LHCII dephosphorylation in *pgr5* leaves while the PSII core proteins became heavily phosphorylated, resulting in concomitant heavy phosphorylation of both the PSII core and LHCII proteins which, in turn, strongly enhanced the relative excitation of PSI (Fig. 4e) as also reported in[21]. The amount of the PSI subunit, PsaH, which provides the site for phosphorylation-induced attachment of P-LHCII to PSI[27], also remained unchanged upon the HL-treatment (Fig. 4e).

***HL-treatment causes an increase in PSI core to LHCI fluorescence ratio in* pgr5 –** To further elucidate the energy transfer properties of photodamaged PSI centres, a Gaussian decomposition of various 77K fluorescence spectral bands was performed[28] (Fig. 5). The fluorescence spectral bands of PSI components showed peaks at 724 nm and 736 nm, known to originate from long wavelength chlorophylls associated with PSI core and LHCI, respectively[29]. HL-treatment of WT plants did not cause any change in relative peak intensities of the PSI core and LHCI (Fig. 5; see supplementary Fig. S2 for relative changes in all time points). In *pgr5,* the changes in fluorescence spectral bands suggested an increase in the PSI core fluorescence with simultaneous decrease in the LHCI fluorescence in the course of HL-treatment of the mutant plants (Fig. 5; Fig. S3). It is thus conceivable that the increase in overall PSI fluorescence at 77 K in *pgr5* indicated both the excess excitation energy flow towards PSI and the quenching of excitation energy by damaged PSI. This provided evidence that the antenna reorganization, increasing the relative excitation of PSI, also led to altered excitation energy transfer inside the "LHCII lake" [20-22]. Such a general rearrangement of the "LHCII lake" is a likely explanation also for the similar changes in the excitation spectra of both PSII and PSI (Fig. S1).

Discussion

Photosynthetic light harvesting and energy conversion mechanisms are potentially dangerous processes both for photosystems themselves and for the surrounding biomolecules. Research on photoprotection mechanisms has so far mainly concentrated on PSII and LHCII. In contrast, PSI has not been considered to be a remarkable target of the photodamage, nor bearing a role in photoprotection. Indeed, the molecular mechanism(s) and physiological role of PSI photoinhibition and the role in photoprotection have remained largely unknown.

PSI is very tolerant against light energy, as long as the electron transfer to PSI is strictly controlled. However, if PSI receives more electrons than the acceptor side can directly handle, the electrons are transferred to molecular oxygen inducing PSI photoinhibition[3, 4, 30]. If PSI acceptor side is sufficiently oxidized to receive an

electron from the excited state of P700 ($P700^*$), the resulting $P700^+$ stays stable until electrons are supplied from PSII (Fig. 1a,c) or slowly from upstream electron transfer cofactors in PSI, as explained below.

The *pgr5* mutant from normal GL exhibits similar amount of functional F_A and F_B clusters as the HL-treated WT thylakoids (Fig. 3b, Supplementary Table S1). Similarly to HL-treated WT, the decreased amount of functional F_A and F_B do not affect the behavior of P700 oxidation in *pgr5*. Despite the remarkable damage of F_A and F_B clusters, the behavior of P700 oxidation remained unaffected (Fig. 1), being in corroboration with earlier report indicating functional charge separation despite the lack of F_A and F_B[31]. This was the case when P700 oxidation was measured *in vivo* in the presence of natural PSI electron acceptors and down-stream metabolism (Fig. 1) but also *in vitro* with thylakoids isolated after the HL- treatment of plants, *i.e.* in the absence of stromal electron acceptor components.

PSI is a heterodimer composed of two subunits, PsaA (A-side) and PsaB (B-side) that differ in their phylloquinone redox potential[32]. Charge separation leads to fast oxidation of P700 in response to forward electron transfer via B-side providing a safe mechanism for charge recombination when electron transfer to PSI is limited[32]. Since the back reactions via A-side are slower than the forward reactions via B-side, P700 remains oxidized in the absence of electron donors under continuous light[32]. The HL-induced damage of F_A and F_B clusters reduces the amount of electron acceptors from F_X, the primary stable electron acceptor. This has severe, but also unexpected consequences on electron transfer in PSI. Indeed, despite the damage of the F_A and F_B clusters, the oxidation of P700 is still occurring, obviously as long as the F_X cluster is present[31]. Thus, the charge separation can occur by fast forward reaction via B-side and the slower back reaction occurring via A-side retains P700 oxidized upon illumination (for explanation see Fig. 6). This explains why P700 can still be oxidized by far red light favouring PSI excitation despite the 50% damage of the F_A and F_B clusters.

When the *pgr5* mutant is exposed to HL, it loses its capacity to oxidize P700 (Figs 1b,d and 6). As explained above, the damage of F_A and F_B alone seems not to lead to the incapability to oxidize P700 by FR light, indicating that further damage of PSI is occurring. The quantification of the F_X clusters failed due to technical problems. Nonetheless, it is highly likely that when the *pgr5* mutant is exposed to HL, also F_X gets damaged, thus abolishing the electron acceptor from the forward reaction and thereby preventing the occurrence of charge separation. Ultimately, under highly reducing environment, charge recombination may take place between A_0^- and $P700^+$ forming a triplet state of P700, which itself is a strong quencher of excitation energy[33]. It is not possible to experimentally define whether P700 is still present in the damaged PSI and just does not get oxidized or whether P700 is degraded. PSI photoinhibition, however, has not been shown to cause photobleaching, which is known to result from release of chlorophyll and subsequent uncontrolled

accumulation of ROS. Rather, the photodamage of PSI was caused by the loss of functions (oxidation/reduction) of the F_A/ F_B clusters and the degradation of PSI proteins (e.g. the PsaA, PsaB, PsaC and PsaD polypeptides shown in Fig. 3c) lags far behind of the photodamage of PSI[2, 5]. The P700 and F_A/ F_B epr signals lost upon photodamage appears partially reversible (presently under detailed EPR investigation). This may indicate that the PSI centres with only F_A and F_B damaged are able to recover, but the damage to F_X leads to permanent loss of PSI activity and eventually to the degradation of PSI centres, which indeed has previously been shown to be a very slow process[2, 5]. It is conceivable that the damage of F_X prevents charge separation but the PSI reaction centre chlorophylls are still present, getting excited/de-excited, and the excitation energy is relaxed via thermal dissipation during de-excitation.

Interestingly, PSI photodamage seems to increase energy transfer to PSI, which however gets normally dissipated. The best known mechanism to increase PSI excitation is "state transition" requiring a use of light quality preferentially exciting PSII and leading to strong reduction of the intersystem ETC, which in turn induces strong activation of both the STN7 and the STN8 kinases [24, 34]. Due to altered redox balance and regulation in the thylakoid membrane of the *pgr5* mutant as compared to WT (Fig. 4c), the HL-treatment induces thylakoid protein phosphorylation in *pgr5* that very much resembles the artificial state 2 in WT, and thus pronouncedly enhances in *pgr5* the distribution of excitation energy to PSI[35]. Thus, the damage of the FeS-clusters in the *pgr5* mutant led to high concomitant PSII core and LHCII protein phosphorylation and strongly increased excitation energy transfer to PSI (Fig. 4c). Therefore, it is evident that the PSI photodamage indirectly, via strong PSII-LHCII phosphorylation, increases the excitation energy transfer to the damaged PSI, which still is able to thermally dissipate the excitation energy. P700 is an efficient thermal dissipator of excitation energy, and its capacity to quench excitation energy is not dependent on its redox state, whether $P700^+$ (under oxidizing conditions) or triplet state of P700 (under highly reducing conditions)[33]. The redox state, however, has a decisive role in deciding the further fate of excitation energy *i.e.* utilization for chemical work vs dissipation as heat. The $P700^+$ reduced by electrons from ETC utilizes all the excitation energy to perform photochemistry whereas upon high excitation and limitation of electron transfer the lifetime of $P700^+$ increases and it thermally dissipates the excess energy without being involved in photochemistry. Therefore, the former is involved in over reduction of the FeS-clusters, and the latter is helping in non-photochemical photoprotective dissipation. The oxidizing or reducing environment further contributes in aforementioned non-photochemical dissipation process by opting between direct dissipation by oxidized P700 or via the triplet state of P700. Both of them are suggested to have an efficient radiationless decay of energy[33]. Photosystem I yields only low fluorescence at physiological temperatures. At the temperature of liquid nitrogen (77 K), PSI is already a strong fluorescence emitter, making it possible to investigate the excitation energy transfer between PSII and PSI. Comparison of fluorescence

emission spectra recorded at RT, where the quenching mechanism is functional, with that recorded at 77 K (Fig. 5c,d), where quenching is severely inhibited, provides information on the function of the quenching mechanisms. As shown in Fig. 5, the damaged PSI centres can still efficiently quench the fluorescence at physiological temperature, indicating that the damage of FeS-clusters is followed by an enhanced capacity of PSI to thermally dissipate excess excitation energy (Fig.5). It indeed seems likely that regardless of the damage of primary electron acceptors of PSI and loss of charge separation, the transfer of excitation energy to reaction centre chlorophylls remains unchanged and is dissipated thermally by P700.

Concluding Remarks

We demonstrate that it is vital to keep P700 oxidized in a continuous basis to protect the PSI FeS-clusters from photooxidative damage upon increase in light intensity (Fig. 6). In order to achieve this, the photosynthetic machinery in plants is endowed with the strong ΔpH-dependent control mechanism of LET from Cyt b_6f to PSI. Concomitantly, excess energy is dissipated by the PSBS- and LHCII-dependent mechanism. However, in the absence of ΔpH, PSI takes a central role in excess energy dissipation and control of LET. The mutant, which fails to execute the controls over LET due to a loss of formation of trans-thylakoid ΔpH (*pgr5*), undergoes gradual damage of FeS-clusters (Fig. 6) to slow down LET and turns on the non-photochemical photoprotective energy dissipation in damaged PSI centres. Non-functional PSI centres thus turn into non-photochemical quenchers (PSI-NPQ) of excitation energy and dissipate thermally the surplus of excitation energy. Moreover, photodamage of PSI increases the antenna cross-section of PSI, and conversely decreases that of PSII, via thylakoid protein phosphorylation dependent mechanisms, thus maximizing the amount of excess energy dissipation by PSI. It is conceivable that the novel transition mechanism of PSI from photochemical to non-photochemical quencher of excitation energy, as demonstrated here, is of remarkable physiological significance, for example, in survival of evergreen plants in nature.

Methods

Growth conditions, light treatment and thylakoids isolation

Arabidopsis thaliana ecotypes Colombia (Col-0) wild type (the trichomeless type *gl1*) and the *pgr5* mutant[11] were grown as described[5]. The HL-treatment of 900 µmol photons m^{-2} s^{-1} was given for 30, 60 and 120 min to detached leaves of plants that had been under growth light for 2-3h. All the experiments were performed with 6-weekold plants.

The chloroplasts were isolated from HL-treated leaves by homogenizing in buffer containing 330 mM sorbitol, 5 mM EDTA, 5 mM EGTA, 5 mM $MgCl_2$, 50 mM Hepes/KOH (pH 7.5), 5 mM sodium ascorbate, 0.5% (w/v) fatty acid-free BSA and 5 mM NaF. The suspension was filtered through 8 layers of Miracloth and centrifuged at 3000×g for 6 min at 4˚C. The pellet was resuspended and washed twice in the buffer containing 100 mM Sorbitol, 10 mM $MgCl_2$, 50 mM Hepes (pH 7.5), and stored at -80˚C. Following storage at -80˚C, the external envelop of chloroplasts was broken and these thylakoids were used further for all the measurements by diluting into the reaction buffer containing 330 mM sorbitol, 10 mM $MgCl_2$ and 50 mM Hepes (pH 7.5).

P700 oxido-reduction measurements

The redox kinetics of P700 was measured from intact leaves and isolated thylakoids (equivalent to 100 µg Chl ml^{-1}) using DUAL-PAM-100 (Walz, Germany). The oxidation of P700 was induced using far red (FR) light and for complete oxidation, a short saturating pulse of actinic light was applied during FR illumination. The kinetics of $P700^+$ re-reduction by intersystem electron transfer pool was determined by using a single turnover (ST) and a multiple turnover (MT) saturating flash in the background of continuous FR light.

EPR measurements

Continuous wave EPR spectra were recorded with an ELEXSYS E500 spectrometer (Bruker Biospin) equipped with a SuperX bridge and a SHQ4122 cavity. For the liquid He temperature measurements the spectrometer was fitted with an Oxford Instruments ESR 900 cryostat and ITC 503 temperature controller. Analysis of the EPR spectra was carried out with Bruker Xepr 2.4b software. EPR measurements at RT were carried out in a flat cell at a Chl concentration of 2.5 mg/ml. PSI/PSII ratio was determined as described in[16]. EPR measurements at liquid He temperatures were carried out in calibrated EPR tubes at a Chl concentration of 5.0 mg ml^{-1}. Spectrometer settings are given in the figure legends. The quantification of P700 and F_A/ F_B clusters was performed using internal standard Tyrosine D and per chlorophyll basis. The $P700^+$ signal at room temperature was induced chemically by addition of 10 mM ferricyanide, which is known to completely oxidize $P700^+$ (100%) [16]. The precision of quantification of both $P700^+$ and F_A/F_B EPR signals are routinely more than 95%. The light inducible F_A/F_B signals at 16K were used for quantification of the FeS-clusters. The amount of light-induced F_A/F_B corresponds to the amount of photo-induced $P700^+$ signal under these conditions, which, reflects the corresponding charge separation at 16K.

Steady state fluorescence measurements

Steady state fluorescence emission spectra were recorded from thylakoids at RT and 77K in reaction buffer as described in[9]. The minimum Chl concentration equivalent to 5 µg ml^{-1} was used to avoid self-absorbance. The Gaussian decomposition of 77 K fluorescence emission spectra were done using SigmaPlot 12.5 software for six Gaussian components having peaks at 680, 685, 695, 700, 720 and 735 nm. The fitting parameters were free running, while some constraints were applied for 720 and 735 nm components to get the best fit with limited variations.

Western blotting

Thylakoid proteins (equivalent to 0,5 µg Chl) were separated with 12% polyacrylamide gels with 6 M urea. Western blotting was performed with standard techniques[26] using P-Thr (New England Biolabs), PsaC (Agrisera,AS10939), PsaH (Agrisera AS06 105 and PsaD antibodies, and the enhanced luminol-based chemiluminescent substrate system (Promega) was used for visualization.

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Footnotes

Authors declare no conflict of interest.

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Legends to Figures

Figure 1. Redox kinetics of PSI upon shifting the WT and *pgr5* plants from growth light to high light for indicated time periods. Leaves were detached from plants 2-3 hr after beginning of the light period and subjected to HL-treatment (900 µmol photons m^{-2} s^{-1}) for 30, 60 and 120 min. Photo-oxidation and subsequent re-reduction of P700 were recorded upon application of single-turnover (ST) and multiple-turnover pulses on continuous background illumination of far-red (FR) light, followed by Pm measurements. **a**, intact leaves of WT; **b**, intact leaves of *pgr5*; **c**, isolated thylakoids from light-treated leaves of WT; **d**, isolated thylakoids from light-treated leaves of *pgr5*.

Figure 2. Room temperature EPR spectra from thylakoid membranes from WT and the *pgr5* mutant. Signal from $Tyr_D^{\bullet}$ **a**, were induced by 30 sec illumination and subsequent dark adaptation for 5 min at RT. Signal from $P700^+$ **b**, was induced by oxidation with 10 mM ferricyanide. Black line spectra are from WT and red line spectra are from *pgr5* mutant. All spectra were measured at the same Chl concentration of 2.5 mg Chl ml^{-1}. Solid line – control samples, dotted line – after 120 min of the HL-treatment. EPR conditions: microwave frequency 9.81 GHz, microwave power 8 mW, modulation amplitude 5 G. **c**, Photoinhibition of the PSII, measured as decrease

of $Tyr_D^{\bullet}$ signal (filled circles) and PSI, measured as decrease of $P700^+$ signal (open circles) in WT (black) and *pgr5* mutant (red). **d**, Kinetic traces of $P700^+$ decay in thylakoid membranes from WT and **e**, *pgr5* mutant induced by laser flash indicated by arrow at 532-400 nm and 400 mJ; Black lines – control, red lines – 30 min, blue lines – 60 min, green lines – 120 min of the HL-treatment. Each trace represents averaging of 8 individual measurements. EPR conditions: microwave frequency 9.81 GHz, microwave power 8 mW, modulation amplitude 5 G, conversion time 41 ms.

Figure 3. Reduction of the F_A and F_B centres of PSI in the thylakoid membrane at 16 K. **a**, WT; **b**, the *pgr5* mutant and **c**, a semiquantitative determination of the PsaC and PsaD proteins by immunoblotting. The gels were loaded on equal chlorophyll basis (100%). Three biological replicates of WT were simultaneously processed for making the quantity controls (50%, 100% and 200%). The EPR signals were induced by illumination with white light for 6 min and spectra shown are light minus dark difference spectra. All spectra were measured at the same Chl concentration of 5 mg Chl ml^{-1}. Black lines – control, red lines – 30 min, blue lines – 60 min and green lines – 120 min of the HL-treatment. The * marks indicate the peaks of the g_z region that are used for quantification. The large absorption from $Tyr_D^{\bullet}$ and $P700^+$ in the radical region (g=2) is omitted for clarity. EPR conditions: microwave frequency, 9.27 GHz, microwave power 20 mW, modulation amplitude 10 G.

Figure 4. **Fluorescence emission spectra from WT and the *pgr5* plants.** Fluorescence emission spectra were measured at RT and 77 K from thylakoids isolated from growth-light-acclimated (2-3 hr) WT and *pgr5* plants and after HL-treatment of 30, 60 and 120 min. The excitation light used was of 440 nm. **a**, WT fluorescence spectra recorded at RT; **b**, *pgr5* at RT; **c**, WT at 77 K; and **d**, *pgr5* at 77 K. All spectra are normalized to 685 nm peak. **e**, Phosphorylation of thylakoid proteins in WT and the *pgr5* mutants in growth light (120 µmol photons m^{-2} s^{-1}) and after exposure to 900 µmol photons m^{-2} s^{-1} for 30 min, 60 min and 120 min. Phosphorylation of thylakoid proteins was determined by immunoblotting with P-Thr antibodies. P-CP43, P-D2, P-D1, and P-LHCII represent phosphorylated forms of the PSII core proteins CP43, D2, and D1 and the LHCII proteins Lhcb1 and Lhcb2. The PsaH immunoblot suggests the unchanged level of LHCII docking site in PSI. The gels were loaded on equal chlorophyll basis (100%). Three biological replicates of WT were simultaneously processed for making the quantity controls (50%, 100% and 200%).

Figure 5. **Gaussian decomposition of low temperature fluorescence emission spectra** of 0 min and 120 min HL-treated thylakoids of the WT and the *pgr5*. The fluorescence was normalized at 735 nm prior to decomposition to sub bands.

Figure 6. **Schematic representation of the regulatory functions of PSI in the presence (a,b)(WT plants) and the absence (c,d)(*pgr5* mutant) of ΔpH-dependent control mechanisms -** Black arrow = controlled electron transfer to PSI; Black arrow in bold = uncontrolled electron transfer to PSI; Red arrow = ≤ growth light (no ΔpH-dependet mechanisms needed); Red arrow in bold = high light (ΔpH-dependet mechanisms needed). **a,** Low excitation and balanced electron flow to PSI in relation to the capacity of stromal electron sinks. Excitation energy limits the electron transfer rate of PSI. **b**, High excitation and controlled electron flow to PSI in relation to the capacity of stromal electron sinks: Efficient ΔpH-dependent mechanisms to limit electron transfer to PSI. Some damage of F_A/F_B clusters may occur (see **c**), but the damage is limited by the induction of PSII photoinhibition and the phosphorylation-dependent mechanism to downregulate PSII (see **d**). Despite the damage of F_A/F_B clusters all P700 remains photooxidable (see details in the text) and $P700^+$ works as a dissipator of excess excitation energy, yet the ΔpH related PSBS protein-dependent LHCII quenching is the main energy dissipation mechanism. **c**, Increase in excitation and failure to control electron flow: P700 gets over reduced due to uncontrolled electron flow from PSII, which rapidly damages the F_A/F_B clusters. Severity of the damage depends on the light intensity and capability of the plant to restore the balance between PSII donor and acceptor side by other mechanisms. **d**, High excitation and uncontrolled electron flow - both P700 and the electron acceptors of PSI become severely over-reduced, which first damages F_A/F_B and eventually F_X clusters. Capability to photooxidise P700 disappers. When the amount of active PSI decreses low enough, the low amount of PSI diminishes the acceptor side limitation and further damage of PSI centres is prevented. Excitation energy transfer to PSI is increased at the expense of PSII excitation and the photoinhibited PSI centres carry out thermal dissipation of the excitation energy via PSI-NPQ. Blockage of electron transfer in PSI leads to high reduction of the intersystem electron transfer chain. This strongly activates the STN7 and STN8 kinases leading to strong concomitant phosphorylation of both the LHCII and PSI core proteins, thus maximizing the excitation energy transfer to PSI at the expense of PSII excitation and the thermal dissipation by PSI.