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PGR5 and NDH-1 systems do not function as protective electron acceptors but mitigate the consequences of PSI inhibition

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ABSTRACT

Avoidance of photoinhibition at photosystem (PS)I is based on synchronized function of PSII, PSI, Cytochrome b_6f and stromal electron acceptors. Here, we used a special light regime, PSI photoinhibition treatment (PIT), in order to specifically inhibit PSI by accumulating excess electrons at the photosystem (Tikkanen and Grebe, 2018). In the analysis, *Arabidopsis thaliana* WT was compared to the *pgr5* and *ndho* mutants, deficient in one of the two main cyclic electron transfer pathways described to function as protective alternative electron acceptors of PSI. The aim was to investigate whether the PGR5 (*pgr5*) and the type I NADH dehydrogenase (NDH-1) (*ndho*) systems protect PSI from excess electron stress and whether they help plants to cope with the consequences of PSI photoinhibition. First, our data reveals that neither PGR5 nor NDH-1 system protects PSI from a sudden burst of electrons. This strongly suggests that these systems in *Arabidopsis thaliana* do not function as direct acceptors of electrons delivered from PSII to PSI – contrasting with the flavodiiron proteins that were found to make *Physcomitrella patens* PSI resistant to the PIT. Second, it is demonstrated that under light-limiting conditions, the electron transfer rate at PSII is linearly dependent on the amount of functional PSI in all genotypes, while under excess light, the PGR5-dependent control of electron flow at the Cytochrome b_6f complex overrides the effect of PSI inhibition. Finally, the PIT is shown to increase the amount of PGR5 and NDH-1 as well as of PTOX, suggesting that they mitigate further damage to PSI after photoinhibition rather than protect against it.

1. Introduction

Photosynthetic energy conversion at thylakoid membrane is based on coordinated function of two light-driven enzymes – the water-oxidizing photosystem (PS)II and the ferredoxin-reducing PSI – as well as the electron transfer chain (ETC) between the photosystems and that beyond PSI. PSII transfers electrons from water via plastoquinone (PQ) pool to Cytochrome (Cyt) b_6f complex that in turn donates electrons to PSI via plastocyanin (PC). Excitation of PSI then initiates the transfer of electrons via ferredoxin (Fd) to Fd–$NADP^+$ oxidoreductase (FNR), which converts $NADP^+$ into NADPH. Strict coordination of each step in the intersystem ETC is essential since the accumulation of excess electrons easily leads to production of reactive oxygen species that damage the FeS centers of PSI [2–5], resulting in a time- and energy-consuming replacement of the whole PSI core [6,7].

In addition to the above described linear electron transfer (LET), two different routes of cyclic electron transfer (CET) have been proposed to function as alternative electron acceptors from PSI and to prevent the over-reduction of the Fd pool by cycling electrons back to the PQ pool. One CET pathway is dependent on the NAD(P)H PQ oxidoreductase, called type I NADH dehydrogenase (NDH-1), and the other on the PROTON GRADIENT REGULATION5 (PGR5) protein (for recent and comprehensive reviews, see e.g. Peltier et al. [8] and Yamori and Shikanai [9]).

NDH-1 has been shown to form supercomplexes with PSI [10,11] and it was recently demonstrated that the NDH-1-dependent CET in rice protects PSI from oxidative damage under fluctuating light [12] and that the NDH-1 is essential also for the normal growth and yield under low light [13]. In addition to CET, in darkness NDH-1 is known to mediate chlororespiration by channeling electrons from NADPH, via Fd and PQ pool, to plastid terminal oxidase (PTOX) [14,15]. PTOX is a small thylakoid protein proposed to function as a safety valve under excess light by draining electrons from the PQ pool to molecular oxygen [16,17], yet the interaction between the dark and the light functions of NDH-1 and PTOX have remained unresolved. Since the electron transfer through NDH-1 is strongly coupled to proton translocation from the

Abbreviations: AL, actinic light; PIT, PSI photoinhibition treatment; FLV, flavodiiron

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Table 1
Light quality, intensity and duration of the targeted PSI photoinhibition treatment for *Arabidopsis thaliana* WT, *pgr5* and *ndho* as well as for *Physcomitrella patens* WT and *flvb*.

Light quality	Light intensity (μmol photons m^{-2} s^{-1})		Duration (s)	Effect on photosynthetic machinery
	Arabidopsis	Physcomitrella		
White	120	30	60	Oxidation of ETC
Red	37	15	5	Excitation of PSII, accumulation of electrons in ETC
White	3250	2500	1	Over-reduction of PSI electron acceptors
Red	37	15	5	Excitation of PSII, accumulation of electrons in ETC
White	3250	2500	1	Over-reduction of PSI electron acceptors
Red	37	15	5	Excitation of PSII, accumulation of electrons in ETC
White	3250	2500	1	Over-reduction of PSI electron acceptors

chloroplast stroma into the thylakoid lumen, the complex is thought to increase ATP production [18].

The PGR5 system is known as the master regulator of photosynthetic energy transduction, enabling plants to cope with changes in light intensity [19–21]. The presumed PGR5-dependent CET has been proposed to work through a small protein complex consisting of the PGR5 and PGRL1 proteins [22]. This view has, however, been questioned due to the lack of solid molecular evidence supporting the CET hypothesis and due to the fact that the *pgr5* mutant is unable to control the proton conductivity of the ATP synthase − a defect that alone is sufficient to explain the mutant phenotype [23].

Since the water oxidation by PSII releases protons into the lumen and the electron transfer via Cyt b_6f is coupled to proton translocation across the thylakoid membrane to the lumen, the function of ETC results in formation of proton motive force, which in turn is a prerequisite for ATP production [24]. The protonation of lumen also serves a regulatory role in preventing the saturation of PSI electron acceptors under excess light by slowing down the proton translocation-coupled electron transfer at Cyt b_6f in a process known as photosynthetic control [25–27]. Moreover, the lumen acidification leads to protonation of PSBS protein, which is involved in thermal dissipation of excess excitation energy from the light harvesting system i.e. in the qE component of the non-photochemical quenching (NPQ) [28]. NPQ keeps the PQ pool optimally oxidized under excess light, but does not modulate the electron transfer from PSII to PSI, which, instead, predominantly remains under the photosynthetic control [21]. Under low light, the accumulation of electrons into the ETC is prevented by the STN7 kinase-dependent phosphorylation of thylakoid proteins [29]. Together the photosynthetic control, NPQ and STN7-dependent LHCII phosphorylation keep the ETC optimally oxidized and thus prevent the saturation of PSI electron acceptors and the consequent PSI photoinhibition upon sudden increases in light intensity [30,31].

Any failure or inadequacy in the regulatory mechanisms controlling the electron transfer to PSI makes PSI extremely prone to photoinhibition. As a fallback, most of the photosynthetic organisms scavenge the excess electrons from PSI to molecular oxygen by flavodiiron (FLV) proteins, producing water [32–34]. Angiosperms have, however, lost these proteins during their early evolution [32,34], which likely enhances the production of reducing power but, simultaneously, increases the risk of PSI photoinhibition. From this perspective, high photosynthetic capacity in angiosperms is achieved only through strict control of ETC. Similarly, the physiological consequences of PSI photoinhibition are largely dependent on their capacity to rebalance the ETC after photoinhibition by controlling and recycling electrons.

To be able to investigate the roles of alternative electron acceptors in the protection of PSI from sudden bursts of electrons, a genotype-independent method for exposing PSI to excess electron stress is essential. For this, we recently introduced a LED lamp-based method that challenges the PSI acceptor side capacity by causing an abrupt burst of electrons from over-reduced ETC to PSI [1]. With *Arabidopsis thaliana* WT, *pgr5* and *ndho*, we utilized this approach here (i) to monitor the capacity of the PGR5 and NDH-1 systems to protect PSI against LET-derived electron bursts, i.e. initially derived from PSII water splitting and (ii) to investigate whether the PGR5 and NDH-1 systems are essential for mitigation of the consequences of PSI photoinhibition. For comparison, we examined *Physcomitrella patens* WT with functional FLV proteins and the *flvb* mutant lacking the FLV proteins [35].

2. Materials and methods

2.1. Growth conditions

5-week-old *Arabidopsis thaliana* (Arabidopsis) WT Columbia and mutant lines *pgr5* [19] and *ndho* [36] were compared in the experiments. The plants were grown at 23 °C and 60% relative humidity under an 8-h photoperiod of constant moderate white light of 100 μmol photons m^{-2} s^{-1} with POWERSTAR HQI-T 400 W/D metal halide lamps (OSRAM GmbH, Munich, Germany) as light source.

Protonemal tissue of *Physcomitrella patens* (Physcomitrella) WT Gransden and mutant line *flvb* [35] were grown for 11 days on solid minimum $PpNO_3$ media at 24 °C under 16-h photoperiod of 50 μmol photons m^{-2} s^{-1}. It is important to note that although *flvb* only lacks the *FLVB* gene, it is strongly affected in the accumulation of both FLVA and FLVB proteins [35].

2.2. PSI photoinhibition treatment

Targeted PSI photoinhibition treatment (PIT) was performed according to [1] for 2 or 4 h for Arabidopsis and for 3 h for Physcomitrella with a series of light treatments described in Table 1. During the PIT, detached leaves of Arabidopsis were floating on tap water in a petri dish, whereas for Physcomitrella, the whole tissue culture was treated. After the PIT, the samples were left to recover for 24 h in their normal growth conditions, where also the control samples were simultaneously kept. As depicted in Fig. 1, after the treatment and recovery, the leaf samples were either subjected to Dual-PAM measurements (Section 2.3), EPR measurements (Section 2.4) or thylakoid isolation (Section 2.5) following western blots or fluorescence measurements at 77 K and 253 K (Sections 2.6 and 2.7).

2.3. Fluorescence and P700 measurements with Dual-PAM

Fluorescence and P700 were recorded with Dual-PAM-100 (Heinz Walz GmbH, Effeltrich, Germany). Chlorophyll a fluorescence was detected with 460-nm measuring light (1 μmol photons m^{-2} s^{-1}) and P700 was determined by the difference of 875-nm and 830-nm measuring lights [37,38]. Before the measurements, the detached leaves were dark-acclimated under measuring light for 10 min. During the program, actinic light (AL) intensity and quality was altered according to Table 2. Dual-PAM measurements for *Physcomitrella patens* were conducted as for Arabidopsis, but with two exceptions: AL illuminations were longer, 3 min, and the red AL intensities smaller,

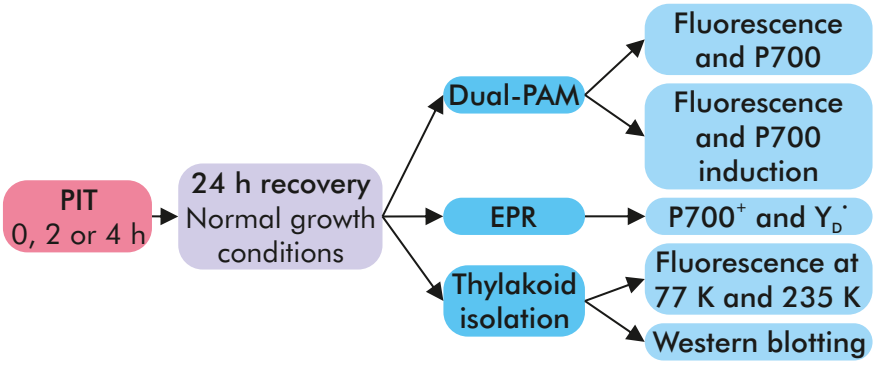

Fig. 1. Experimental procedure. After 0, 2 and 4 h of PSI photoinhibition treatment (PIT), the treated leaves were allowed to rebalance their ETC under their normal growth light for 24 h. Subsequently, the leaves were subjected to Dual-PAM and EPR measurements. Additionally, thylakoids were isolated in order to record fluorescence at 77 and 253 K and to quantitate certain proteins with western blotting.

25 μmol photons m^{-2} s^{-1} for low and 400 μmol photons m^{-2} s^{-1} for high AL. Slow kinetics data was normalized to the first and the fast kinetics data to the last data point. Statistical significance among the P_M and F_M values as well as among the parameters was determined with one-way ANOVA and Tukey's test ($p < 0.01$ and $p < 0.05$).

2.4. Quantification of PSI and PSII by EPR in leaves

For quantification of PSI and PSII in Arabidopsis leaves, measurements of oxidized tyrosine D (Y_D·) and oxidized P700 ($P700^+$) were performed on freshly cut leaf strips using a tissue cell holder on Miniscope (MS5000) EPR spectrometer. First, the detached leaves were incubated in 50 μM DCMU in darkness for 30 min. The infiltration of DCMU into the intact leaves was confirmed by a complete inhibition of P700 re-reduction measured with Dual-PAM using a saturating pulse of AL under background of far-red light. Leaf strips of 5 mm × 20 mm size were cut from the DCMU-infiltrated leaves and kept floating on DCMU solution until the measurements. The EPR measurements of $P700^+$ were performed under continuous illumination of 800 μM photons m^{-2} s^{-1} by Hamamatsu light source (LC8) on leaf strip placed in EPR tissue cell. The DCMU infiltration in leaves generated maximal $P700^+$ accumulation during illumination as observed also in the Dual-PAM measurements and this was assumed to consist of one spin per PSI reaction center. After the measurements in the light and following 2 min of dark incubation, Y_D· D signal was recorded in the same leaf strips in the dark. The individual signal of $P700^+$ was deconvoluted by subtracting the post-illuminated dark spectrum (consisting only of Y_D·) from the light spectrum (consisting of both Y_D· and $P700^+$) as demonstrated earlier [39]. Relative concentrations of Y_D· and $P700^+$ were determined by double integration of the deconvoluted $P700^+$ and Y_D· spectra. The EPR settings used for measurements were as follows: frequency 9.4 gHz, center field 336 mT, field sweep ± 5 mT, modulation frequency 100 kHz with modulation width of 0.5 mT and microwave power 2 mW.

2.5. Thylakoid isolation

Thylakoid membranes were isolated from control and photoinhibition-treated leaves. First, the leaves were ground in ice-cold grinding buffer (50 mM Hepes-NaOH pH 7.5, 330 mM sorbitol, 5 mM $MgCl_2$, 0.05% (w/v) BSA and 10 mM NaF) and filtered through Miracloth (Millipore). Chloroplasts were collected by centrifugation at with 3952 ×*g* for 7 min at 4 °C and ruptured osmotically in ice-cold shock buffer (50 mM Hepes-NaOH pH 7.5, 5 mM sorbitol, 10 mM $MgCl_2$ and 10 mM NaF). The released thylakoid membranes were collected by centrifugation at 3952 ×*g* for 7 min at 4 °C and suspended in storage buffer (50 mM Hepes-NaOH pH 7.5, 100 mM sorbitol, 10 mM $MgCl_2$ and 10 mM NaF) on ice. Chlorophyll (chl) concentration was determined according to Porra et al. [73].

2.6. Western blotting

The isolated thylakoids were diluted first into concentration of 2 μg chl/μl with storage buffer and further into 1 μg chl/μl with sample buffer (138 mM Tris–HCl pH 6.8, 6 M urea, 22.2% (v/v) glycerol, 4.3% (w/v) SDS, 10% (v/v) b-mercaptoethanol), after which the soluble material was collected by centrifugation. The ready samples were run on SDS-PAGE containing 15% acrylamide and 6 M urea, and the separated proteins were subsequently transferred onto PVDF membrane (Millipore). Phosphorylated threonine residues were recognized with antibody from New England Biolabs (catalogue number 6949S); D1, PGR5 and PGRL1 proteins with antibodies from Agrisera (catalogue numbers AS10704, AS163985 and AS10725); and PSAD, NDHL and PTOX proteins with antibodies kindly provided by Poul Erik Jensen, Toshiharu Shikanai and Marcel Kuntz. The primary antibody signal was then detected with horseradish peroxidase-linked secondary antibody (Agrisera) and Amersham ECL Western blotting detection reagents (GE Healthcare). Finally, all proteins were visualized with 0.1% Coomassie Brilliant Blue diluted into 40% (v/v) methanol and 10% (v/v) acetic acid.

Table 2
Light quality, intensity, duration and effect of the Dual-PAM program used in the measurements.

Light quality	Light intensity (μmol photons m^{-2} s^{-1})	Duration (s)	Effect on photosynthetic machinery
Measuring beam	1	600	Relaxation of photosynthesis
F_M-determination pulse	6000	0.7	Maximal reduction of PSII
P_M-determination pulse	6000	0.7	Maximal oxidation of PSI
Darkness	0	60	Relaxation of photosynthesis
Far-red actinic light	62	60	Oxidation of ETC
Saturating pulse	6000	0.7	Saturation of both PSII and PSI
Darkness	0	60	Relaxation of photosynthesis
Red actinic light	50	60	Reduction of ETC
Saturating pulse	6000	0.7	Saturation of both PSII and PSI
Darkness	0	60	Relaxation of photosynthesis
Red actinic light	600	60	Reduction of ETC and induction of photosynthetic control and NPQ
Saturating pulse	6000	0.7	Saturation of both PSII and PSI

2.7. Fluorescence measurements at 77 K and 253 K

According to their chl concentration, the isolated thylakoids were diluted with the storage buffer into final concentration of 2 μg chl/100 μl. Fluorescence spectra were acquired with Ocean Optics S2000 spectrometer using 480 nm excitation light and, during the measurements, the samples were kept in −20 °C block (253 K) or submerged in liquid nitrogen (−196 °C, 77 K). Data were normalized to 685 nm.

3. Results

3.1. Susceptibility of WT, pgr5 and ndho to PSI photoinhibitory treatment

In order to investigate the capacity of the PGR5 and NDH-1 systems to alleviate the excess electron pressure on PSI, *Arabidopsis thaliana* (Arabidopsis) WT, *pgr5* and *ndho* leaves were exposed to PSI photoinhibition treatment (PIT). The treatment is based on repetitive excess electron burst towards PSI electron acceptors [1] and is described in detail in Table 1. After the PIT, the leaves were returned into their normal growth conditions for 24 h, which allowed enough time to rebalance their photosynthetic machinery according to the new PSI-limited state without any substantial recovery in the amount of active PSI [1]. As depicted in Fig. 1, it is important to note that all the experiments were conducted after this 24-h acclimation period.

The amount of functional PSI and PSII were determined by measuring P_M and F_M with Dual-PAM (Fig. 2A) as well as $P700^+$ and tyrosine D radical (Y_D·) with EPR (Fig. 2B). In addition, the total amount of PSI and PSII were visualized by immunodetection of reaction center subunits PSAD and D1, respectively (Fig. 2C). After the 2-h PIT, P_M decreased into about 60, 50 and 50% in WT, *pgr5* and *ndho*, respectively, whereas the 4-h PIT induced a further decline into 30, 15 and 25% of the pre-treatment values. $P700^+$, on the other hand, decreased into 45, 60 and 55% after the 2-h inhibition and into 40, 30 and 35% of the control levels after the 4-h PIT. While the both PSI indicators (P_M and $P700^+$) were greatly affected by the PIT, the PSII parameters (F_M and Y_D·) remained unchanged, confirming that the PIT specifically inactivates PSI, leaving the function (Fig. 2A and B) and amount (Fig. 2C) of PSII unaffected. Notably, the PIT inhibited only the function of PSI (Fig. 2A and B), while the total amount of PSI proteins remained unaffected during PIT in WT and *pgr5*, and even increased in *ndho* (Fig. 2C). In line with earlier reports [5,20], the amount of functional PSI was lower in *pgr5* than in WT and *ndho* already before PIT. Despite the decreased amount of functional PSI before PIT, the relative functionality of PSI was affected rather similarly by PIT in all genotypes, indicating that neither the PGR5 nor the NDH-1 system is able to protect PSI from electrons that have accumulated in the ETC due to an excitation imbalance.

To understand the photoprotective function of alternative PSI electron acceptors, we next conducted some comparative experiments with moss *Physcomitrella patens* (Physcomitrella) WT, which harbors a specific electron transfer pathway from PSI to molecular oxygen by flavodiiron (FLV) proteins FLVA and FLVB, and the *flvb* mutant, which lacks the FLV proteins altogether [35]. As Arabidopsis, Physcomitrella WT and *flvb* were subjected to the PIT-induced electron stress and allowed to rebalance their photosynthetic machinery for 24 h. After the recovery, the amount of functional PSI and PSII were determined with Dual-PAM parameters P_M and F_M, and, as in Arabidopsis, PIT left the PSII of Physcomitrella unaffected (Supplemental Fig. 1). Strikingly, and contrary to Arabidopsis, the PSI of Physcomitrella WT did not reveal any notable PSI photoinhibition. Instead, the Physcomitrella *flvb* mutant showed a significant decrease in P_M as a response to the PIT – similarly to Arabidopsis WT, *pgr5* and *ndho*. This indicates that, unlike the PGR5 and NDH-1 systems in Arabidopsis, the FLV proteins of Physcomitrella were able to protect PSI against photodamage upon the burst of electrons.

Next, with an aim to examine the effect of PSI photoinhibition on

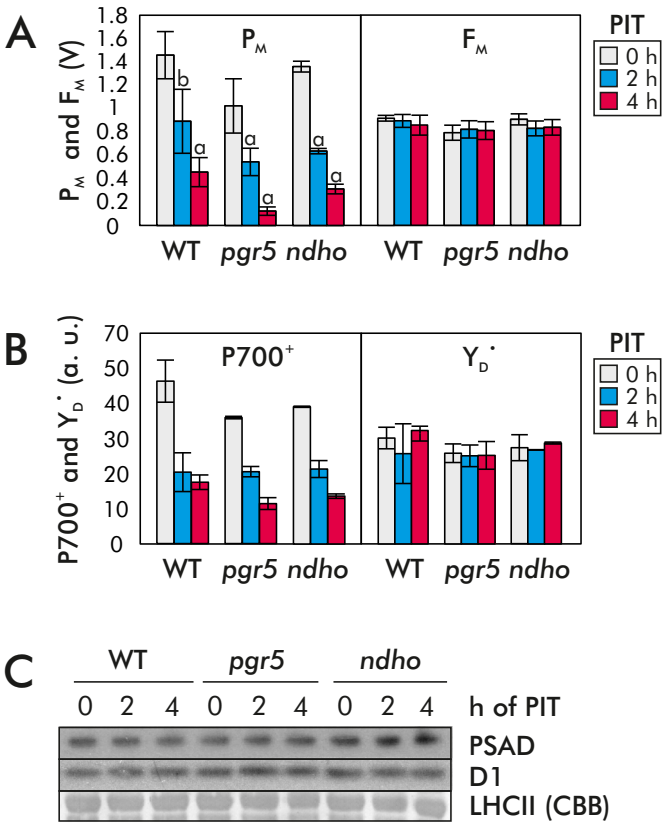

Fig. 2. Function and amount of photosystems after PSI photoinhibition. (**A**) Dual-PAM variables P_M and F_M as well as (**B**) EPR signals $P700^+$ and Y_D· were determined from *Arabidopsis thaliana* WT, *pgr5* and *ndho* leaves after 0, 2 and 4 h of PSI photoinhibition treatment (PIT). Averages of 3–5 measurements are shown with their standard deviations. (**C**) The proteins of isolated thylakoid membranes of *Arabidopsis thaliana* WT, *pgr5* and *ndho* after 0, 2 and 4 h of PIT were separated with SDS-PAGE, transferred onto PVDF membrane and probed with antibodies against PSAD and D1, representing the amount of PSI and PSII, respectively. Equal sample loading was confirmed by visualizing LHCII with Coomassie Brilliant Blue (CBB). Before any experiments, the treated samples were allowed to rebalance their photosynthetic machinery for 24 h under growth light conditions and, prior to the Dual-PAM measurements, the leaves were dark-acclimated for 10 min.

the transfer of excitation energy from the light harvesting system to the photosystems, fluorescence emission spectra from isolated Arabidopsis thylakoids were recorded at 77 K and 253 K (Fig. 3A). At 253 K, PSI efficiently dissipates any excess excitation energy and consequently emits practically no fluorescence, but when the temperature is decreased to 77 K, the dissipation mechanisms are largely prevented, and PSI becomes a strong fluorescence emitter. For this reason, the difference between the 253 K and 77 K fluorescence spectra can be used as an estimation for the thermal dissipation capacity of PSI [5].

At 77 K, all genotypes showed an increase in relative excitation of PSI (733 nm) after the 2 h of PIT, most prominently in WT (Fig. 3A). After the 4-h PIT, the fluorescence emission from PSI further increased in *pgr5* and *ndho*, whereas the emission in WT reached its maximum already after the 2-h PIT. At 253 K, on the contrary, the inhibition of PSI caused no changes in fluorescence in any genotype (Fig. 3A). The increase in temperature from 77 to 253 K substantially decreased the PSI fluorescence in all genotypes independently of the duration of the PIT, indicating that the PIT had no effect on the capacity of PSI to dissipate excitation energy. Notably, no indication of free LHCII trimers (680 nm) or aggregated LHCII (700 nm) was detected, ensuring that the antenna system remained intact and connected to the photosystems.

The excitation energy distribution between PSII and PSI is regulated

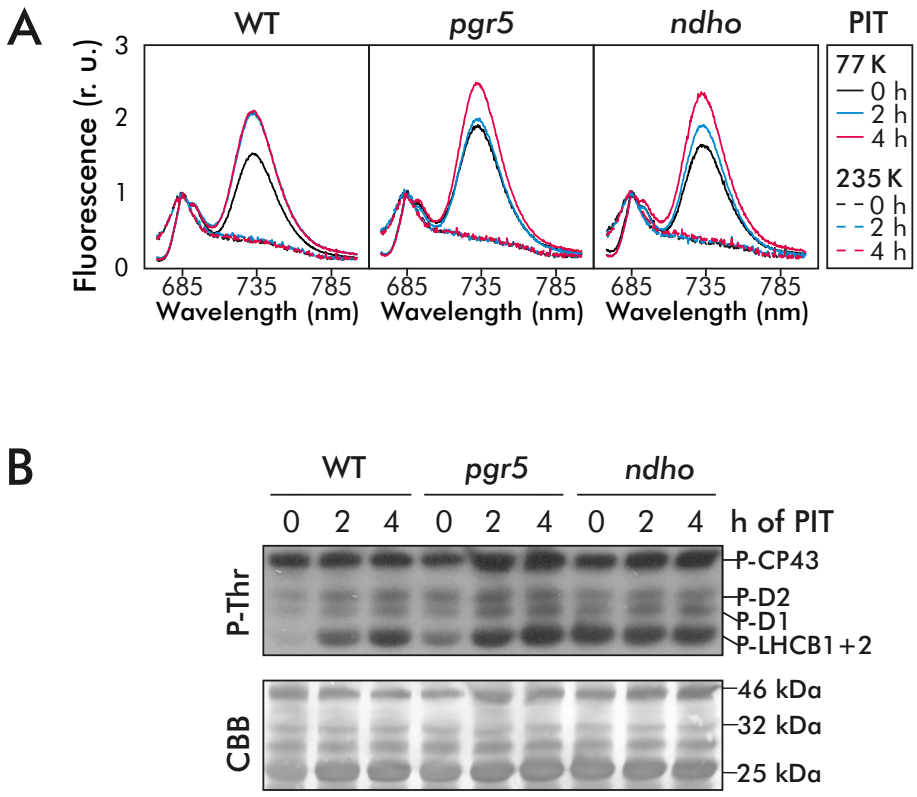

Fig. 3. The relative excitation of photosystems and the phosphorylation status of thylakoid proteins after PSI photoinhibition. (**A**) Fluorescence emission at 77 K and 253 K was recorded from *Arabidopsis thaliana* WT, *pgr5* and *ndho* thylakoid membranes isolated from leaves subjected to 0, 2 and 4 h of PSI photoinhibition treatment (PIT) and diluted into 2 μg chl/100 μl. Spectra were normalized to 685 nm and averages of 3 measurements are shown. (**B**) Intensity of thylakoid protein phosphorylation was determined with P-Thr antibody [72] from thylakoid proteins that were isolated from the same WT, *pgr5* and *ndho* leaves as above, separated with SDS-PAGE and transferred onto PVDF membrane. Equal sample loading was confirmed with Coomassie Brilliant Blue (CBB) staining. Before the experiments, the treated leaves were allowed to rebalance their photosynthetic machinery for 24 h under growth light conditions.

by redox-controlled thylakoid protein phosphorylation and for this reason, in addition to the fluorescence spectra, also the phosphorylation status of the thylakoid proteins was detected (Fig. 3B). As the PSI photoinhibition proceeded, the electrons accumulating in the intersystem ETC activated the STN7 and STN8 kinases and, as a result, the phosphorylation of LHCII as well as the PSII core proteins was enhanced.

3.2. PSI photoinhibition increases the rate of electron transfer via still active photosystems

To investigate the effect of PSI photoinhibition on the capacity of electron transfer (ETR) through PSII and PSI in Arabidopsis WT, *pgr5* and *ndho*, the Dual-PAM parameters ETR(II) and ETR(I) were determined from the fluorescence and P700 values during the saturating pulse given at the end of 1-min low and 1-min high red actinic light (AL) (Fig. 4). Dual-PAM calculates ETR(II) as $[(F_M' - F')/F_M'] \times PPFD \times absorptance \times d_{II}$, and, similarly, ETR(I) as $[(P_M' - P)/P_M] \times PPFD \times absorptance \times d_I$. The absorptance is assumed as 0.84, whereas the d_{II} and d_I i.e. the fraction of absorbed light distributed to PSII and PSI, are both assumed as 0.5. As demonstrated in Fig. 3A, the relative distribution of energy towards PSII (d_{II}) decreased and towards PSI (d_I) increased as a response to PIT, i.e. parallel with the respective ETR parameters, and therefore, the d_{II} and d_I were left as the default value 0.5.

As expected, ETR(II) decreased in all genotypes when the number of PSI began to limit the electron transfer after the 1-min low AL (Fig. 4A). After the 1-min high AL, on the other hand, the PIT had no effect on ETR(II) in WT and *ndho*, but continued to decrease in *pgr5*, demonstrating that, under excess light, NPQ and photosynthetic control modulate the rate of electron transfer at PSII more than does the capacity of PSI to accept electrons. Contrary to ETR(II), ETR(I) interestingly increased in all genotypes as a response to the PIT after the 1-min low AL. After the 1-min high AL, the electron transfer through PSI continued to increase in WT and *ndho*, whereas remained unchanged in *pgr5* (Fig. 4B). The behavior of ETR(I) suggest that, in the case of PSI photoinhibition, plants are capable of accelerating the electron transfer through the still functional PSI centers with a mechanism dependent on the PGR5 system. As to Physcomitrella, PIT did not have any effect either on ETR(II) nor ETR(I) in WT, whereas the *flvb* mutant behaved similarly to Arabidopsis WT (Supplemental Fig. 2).

To clarify the factors connecting the photoinhibited PSI to the increased ETR(I) in the presence of PGR5, we next subjected the Arabidopsis WT, *pgr5* and *ndho* after 0, 2 and 4-h PIT, to specific 1-min light conditions that differently challenge the functional balance of ETC (Quality and quantity of light, see Table 2). Far-red AL was used to favor PSI excitation, low red AL to moderately excite both photosystems and high red AL to strongly excite both photosystems and to concomitantly activate the photosynthetic control and NPQ. Again, the Dual-PAM parameters were calculated from the fluorescence and P700 values during the saturating pulse given at the end of each 1-min light period.

First, the donor side limitation i.e. the oxidation state of PSI after each 1-min light period was determined with the parameter Y (ND) = P/P_M and the acceptor side limitation of PSI with the parameter Y(NA) = $P_M–P_M'/P_M$ [37,38]. As shown by the descending Y(ND) in Fig. 5A, PSI photoinhibition strongly decreased the capacity of far-red light to oxidize P700 in all genotypes. Intriguingly, as compared to WT, the effect of PIT on the far-red-light-driven oxidation of P700 was weakened in *pgr5* and, conversely, strengthened in *ndho*. This might indicate that the action of NDH-1 starts to limit the electron transfer to PSI as a result of PSI photoinhibition and that this mechanism is enhanced in the *pgr5* mutant (see Discussion). Under low AL, the PIT further decreased the already rather low Y(ND) parameter of the PSI centers in WT and *ndho*, while the residual oxidation in *pgr5* completely disappeared. Under high AL, *pgr5* was expectedly incapable of oxidizing P700, but, interestingly, PIT increased the oxidation level of P700 in WT and especially in *ndho*, demonstrating that, in the presence of photosynthetic control, the still functional PSI centers are capable of enhancing their oxidation. In Physcomitrella, the *flvb* mutant showed

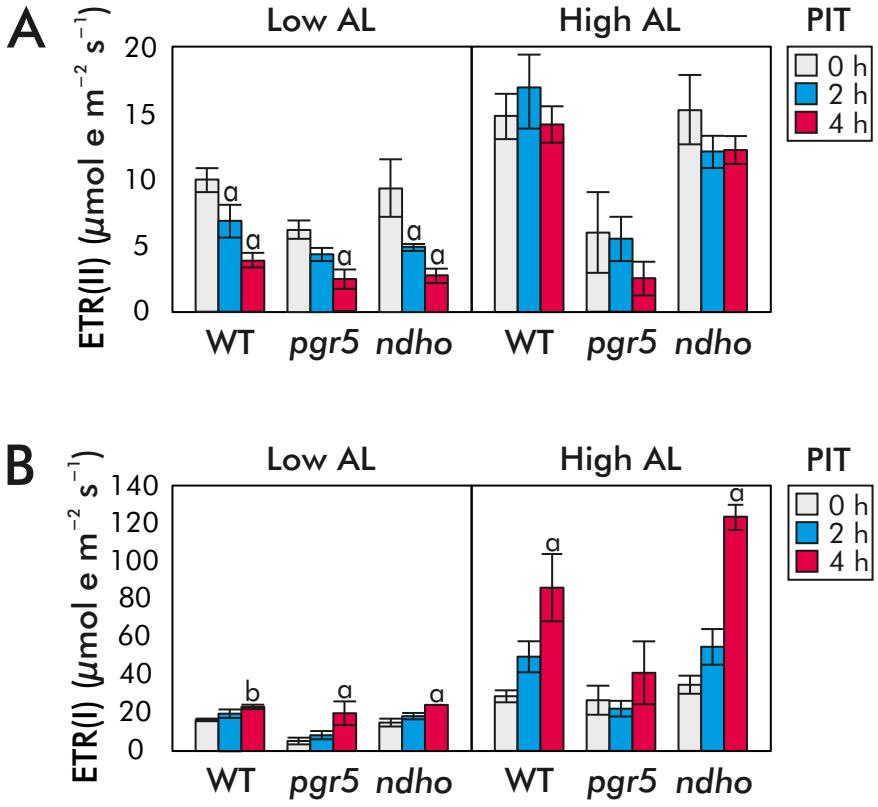

Fig. 4. Electron transfer through photosystems after PSI photoinhibition treatment. Electron transfer rate (ETR) through PSII and PSI in *Arabidopsis thaliana* WT, *pgr5* and *ndho* leaves after 0, 2 and 4 h of PSI photoinhibition treatment was determined with Dual-PAM parameters (**A**) ETR(II) and (**B**) ETR(I), respectively, using 1 min of low (50 µmol photons m^{-2} s^{-1}) or high (600 µmol photons m^{-2} s^{-1}) actinic light (AL) illumination. After the PSI photoinhibition treatment, the leaves were allowed to rebalance their photosynthetic machinery for 24 h under growth light conditions and prior to the Dual-PAM measurements, the leaves were dark acclimated for 10 min. Averages of 3–5 measurements are shown with their standard deviations.

an increased Y(ND) in low AL as compared to WT (Supplemental Fig. 3A), indicating an enhanced control of electron transfer to PSI. After the PIT, however, PSI became the limiting factor of electron transfer and the Y(ND) of *flvb* decreased (Supplemental Fig. 3A).

Since it is well known that the introduction of alternative electron acceptors is able to enhance the photo-oxidation of P700 by alleviating the acceptor side limitation of PSI in saturating light conditions [40], we next examined the parameter Y(NA) of Arabidopsis to test whether the accelerated P700 oxidation after PSI inhibition results from a better availability of electron acceptors per undamaged PSI. As expected, far-red AL did not cause any acceptor side limitation in any genotype before or after the PIT (Fig. 5B). Although the low AL was not expected to be able to saturate the PSI electron acceptors, some acceptor side limitation was detected even in WT and *ndho*, but especially in *pgr5* (Fig. 5B), likely due to the fact that the red AL of Dual-PAM favors PSII over PSI and consequently accumulates electrons, which then exceed the capacity of PSI electron acceptors during the saturating pulse. It is important to note that such a condition, similar to PIT, is dangerous to PSI and, for this reason, the possibility of PSI inhibition should be taken into account in any PAM application combining low PSII-favoring AL and saturating pulses. The fact that the PSI of *pgr5* was strongly acceptor side-limited after the 1-min low AL (Fig. 5B) indicates that PGR5 is required to limit the electron transfer from PSII to PSI even under low light (Fig. 4A), given that the stromal metabolism is not yet fully activated, as was the case in the dark-to-light transition in our Dual-PAM program (Table 2). Under steady state low light conditions, however, PGR5 is not needed [41]. Interestingly, the decrease in the amount of PSI was enough to alleviate the acceptor side limitation of *pgr5* under low AL (Fig. 5B), suggesting a delicate interaction between the relative capacities of PSII and PSI and the PGR5-dependent control of electron transfer from PSII to PSI. Under high AL, WT showed only minor, *ndho* mutant moderate and *pgr5* very strong acceptor side limitation of PSI before the PIT (Fig. 5B). When the number of functional PSI centers was decreased under 30% in WT and 25% in *ndho* (P_M in Fig. 2A), also Y (NA) vanished, indicating that PSI inhibition really alleviates the acceptor side limitation of PSI. In *pgr5*, however, even the 4-h PIT was insufficient to relieve the acceptor side limitation under high AL (Fig. 5B).

Altogether, the behavior of the Y(NA) parameter demonstrated that, in Arabidopsis, the declined number of PSI centers increases the capacity of electron acceptors in relation to the capacity of PSI and thus relieves the acceptor side limitation of the still functional PSI centers. The comparative experiments with Physcomitrella *flvb* mutant showed a decreased Y(NA) in low AL (Supplemental Fig. 3B), together with an elevated Y(ND), indicating that the mutant is able to keep ETC more oxidized than WT. Unlike in Arabidopsis, the PIT in Physcomitrella resulted in an increase in Y(NA), demonstrating that, when PSI becomes the limiting factor of ETC, the electrons accumulated in ETC exceed the capacity of PSI electron acceptors during a saturating pulse. The distinct behavior of Y(ND) and Y(NA) indicate that Arabidopsis is able to increase the acceptor side capacity of the remaining PSI centers after PSI inhibition, whereas Physcomitrella does not possess such plasticity.

Next, the consequences of PSI photoinhibition on the relative redox state of PQ as well as on the NPQ induction were examined by monitoring the fluorescence parameters F'/F_M and $1 - (F_M'/F_M)$ of Arabidopsis, respectively (Fig. 5C and D). As the inhibition of PSI proceeded, Q_A got gradually reduced in all genotypes even under far-red AL, which only marginally excites PSII. Nevertheless, the PIT affected the Q_A redox state more prominently under low AL, especially in *pgr5* (Fig. 5C). Under high AL, on the other hand, the photosynthetic control and NPQ were able to prevent the over-reduction of ETC in WT and in *ndho*, whereas *pgr5*, lacking these mechanisms, kept ETC reduced under both the low and high AL intensities. The NPQ induction (Fig. 5D) remained unaffected during the PSI photoinhibition under far-red AL ($F_M' \approx F_M$). In WT and *ndho*, this was the case also under high AL due to the high-light induced NPQ, whereas in the absence of this mechanism in *pgr5*, the PIT decreased the induction of NPQ. Interestingly, in the *flvb* mutant of Physcomitrella, the effect of PIT on the fluorescence parameters resembled that in Arabidopsis WT (Supplemental Fig. 3). It is, however, important to note that, in Physcomitrella,

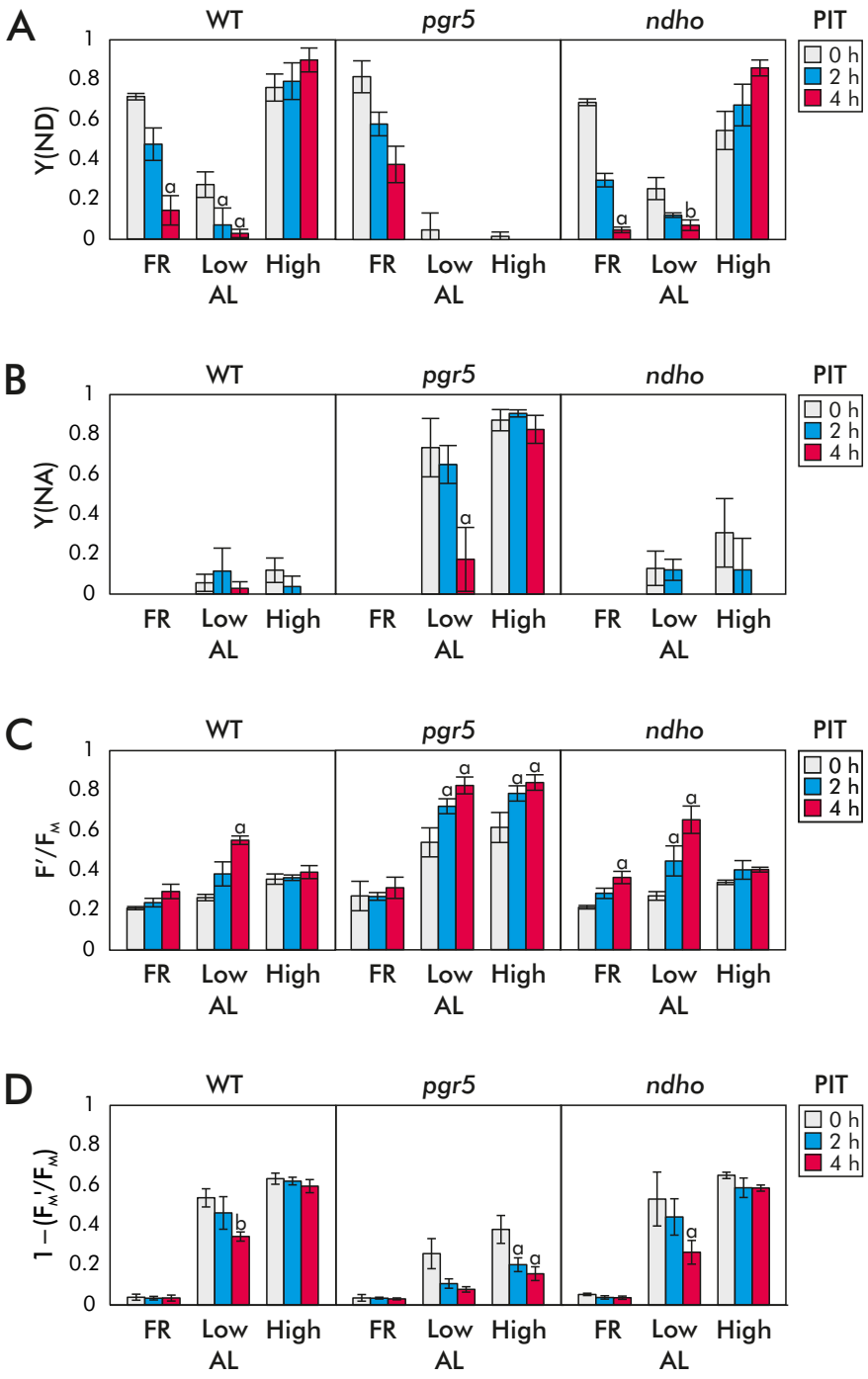

Fig. 5. Dynamics of redox state of the electron transfer chain in PSI photoinhibited leaves after PSI photoinhibition treatment (PIT). Dual-PAM parameters (**A**) Y (ND) = P/P_M (PSI donor side limitation or P700 oxidation), (**B**) Y(NA) = $(P_M - P_M')/P_M$ (PSI acceptor side limitation), (**C**) F'/F_M, (relative Q_A reduction) as well as (**D**) $1 - (F_M'/F_M)$ (NPQ induction) were calculated from *Arabidopsis thaliana* WT, *pgr5* and *ndho* leaves (with 0, 2 and 4 h of PIT) using 1 min of actinic light (AL) illumination comprised of far-red (FR), low intensity (50 μmol photons m^{-2} s^{-1}) and high intensity (600 μmol photons m^{-2} s^{-1}) illumination. After the PIT, the leaves were allowed to rebalance their photosynthetic machinery for 24 h under growth light conditions and, prior to the Dual-PAM measurements, the leaves were dark-acclimated for 10 min. Averages of 3–5 measurements are shown with their standard deviations. Statistical significance between the samples was determined with one-way ANOVA and Tukey's test, and significant differences between the control (0 h) and photoinhibited (2 h and 4 h) samples are shown with letters a and b, referring to $p < 0.01$ and $p < 0.05$, respectively.

the high reduction of Q_A (Supplemental Fig. 3C) resulted in high Y(NA) (Supplemental Fig. 3B), whereas Arabidopsis was able to dispose these excess electrons (Fig. 5B and C).

Altogether the results in Fig. 5 and Supplemental Fig. 3 indicate that, in both Arabidopsis and Physcomitrella, PSI inhibition easily becomes the limiting factor of electron transfer under low light. Under high light, on the other hand, only a small fraction of functional PSI is sufficient to saturate the stromal acceptor capacity, and, instead, the PGR5-dependent photosynthetic control represents the main limiting factor of electron transfer. The results also indicate that the FLV proteins of Physcomitrella function in preventing the acceptor side limitation of PSI upon high light peaks in the PSI-limited state, whereas Arabidopsis possess a high capacity to utilize the electrons independently of the FLV proteins.

3.3. P700 oxidation during saturating pulse requires preceding light-activation of the PGR5 system

As demonstrated above, the inhibition of PSI leads to accumulation of electrons in the ETC in both Arabidopsis and Physcomitrella. These electrons are potentially dangerous for the remaining PSI upon any additional increase in light intensity. In order to avoid any further electron transfer from the over-reduced PQ pool, it is essential to activate the photosynthetic control very rapidly upon any increase in light intensity. To get more insights into the induction of the photosynthetic control in the different AL conditions as well as into its dependency on the number of active PSI centers and the PGR5 and NDH-1 systems, we next recorded the induction of the fluorescence and P700 signals. To this end, Arabidopsis WT, *pgr5* and *ndho* after the 0, 2 and 4-h PIT were

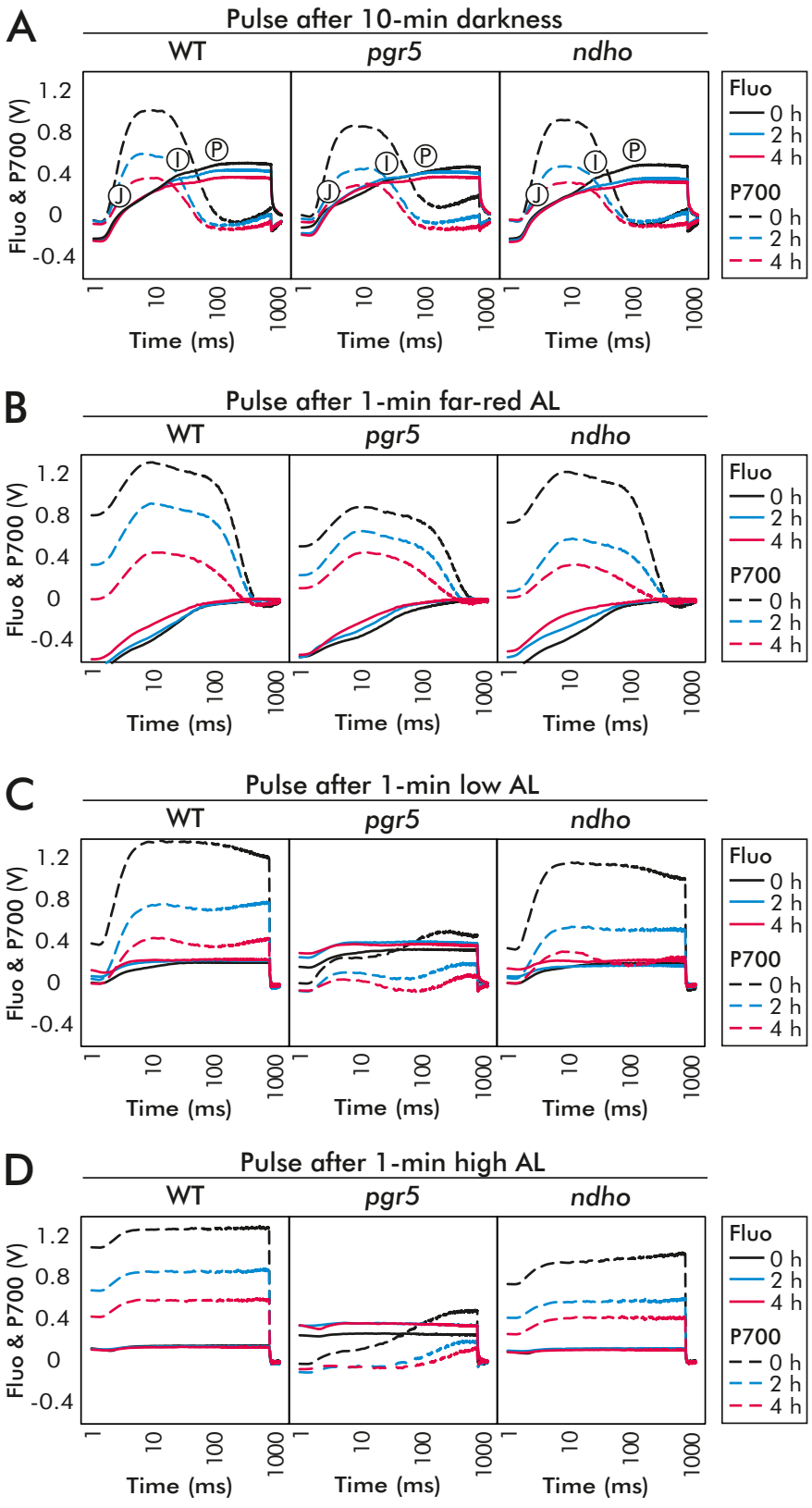

Fig. 6. Effect of PSI photoinhibition treatment and different background illuminations on PSII and PSI redox state during saturating pulse. Kinetics of fluorescence induction (solid lines) and P700 oxidation (dashed lines) from *Arabidopsis thaliana* WT, *pgr5* and *ndho* leaves (0, 2 and 4 h of PSI photoinhibition treatment) were determined with Dual-PAM after (**A**) 10 min dark acclimation and after 1 min of (**B**) far-red, (**C**) low (50 μmol photons m^{-2} s^{-1}) and (**D**) high (600 μmol photons m^{-2} s^{-1}) actinic light (AL). The J-I-P transitions of fluorescence rise are marked with respective letters [43]. After the PSI photoinhibition treatment, the leaves were allowed to rebalance their photosynthetic machinery for 24 h under growth light conditions and, prior to the Dual-PAM measurements, the leaves were dark-acclimated for 10 min. Data are average of 3–5 independent measurements and were normalized to the final value at 1600 ms. Curves are presented on $\log_{10}$ scale.

treated with 10 min of darkness (NDH-1-dependent dark-reduction of ETC), 1 min of far-red AL (oxidized ETC), 1 min of low AL (reduced ETC) and 1 min of high AL (photosynthetic control and NPQ activated) and, after each light step, a saturating pulse was given (Fig. 6).

The first saturating pulse was given after the 10-min dark acclimation, during which the redox state of ETC is determined by the NDH-1-dependent reduction and the PTOX-dependent oxidation of PQ [42]. During this first pulse, the fluorescence induction in the 0-h PIT leaves of all genotypes followed the O-J-I-P behavior [43,44] (Fig. 6A). However, as the amount of functional PSI decreased in the course of the PIT, the J-I and I-P transitions flattened in WT and *ndho*, and the I-P in *pgr5*, indicating an enhanced electron transfer from PSII. The lowered J-

I and I-P transitions in combination with the high F'/F_M (Fig. 5C) suggest that the PSI inhibition blocks a fraction of the ETCs (high F'/F_M), but concomitantly facilitates the function of the PSII pool that is not affected by the inhibited PSI (flat J-I and I-P transition). As to the comparative experiments with Physcomitrella, the fluorescence induction was not affected by the lack of FLV proteins in the control samples, whereas the effect of PIT on the *flvb* mutant resembled that of WT Arabidopsis (Supplemental Fig. 4).

Fluorescence kinetics during the saturating pulse recorded after 1-min far-red AL, which favors PSI and thus oxidizes ETC, remained very low in Arabidopsis at the beginning of the curve (Fig. 6B). PIT, however, resulted in higher fluorescence emission in all genotypes of Arabidopsis and also in the *flvb* mutant of Physcomitrella. As already explained above with the parameters Y(ND) and F'/F_M (Fig. 5A and C), this suggests that the characteristic of far-red light to specifically excite PSI strongly depends on the functional PSI to PSII ratio. Moreover, this implies that the consequences of the PSI inhibition override the possible effect of PGR5 and NDH-1 under far-red light. Contrary to the pulses after darkness and far-red AL, the PIT caused no drastic alterations in the fluorescence induction after the 1-min low or high AL (Fig. 6C and D). The lack of NPQ in *pgr5* was, however, evident on the slightly higher fluorescence emission at the beginning of the pulse, as compared to WT and *ndho* (Fig. 6C and D).

As to the P700 oxidation after the 10-min dark acclimation, P700 rapidly oxidized and thereafter started to re-reduce in all Arabidopsis genotypes (Fig. 6A). After the 1-min far-red light acclimation, on the other hand, P700 was already seen largely oxidized and the saturating pulse was able to only moderately increase the P700 oxidation level in all genotypes (Fig. 6B). As compared to the kinetics after the 10-min darkness, P700 remained longer in the oxidized state after the 1-min far-red AL, yet was eventually similarly reduced as after the darkness. Both after the 10-min darkness and the 1-min far-red AL, PIT was able decreased the amplitude of the signal, but failed to substantially affect the kinetics, which were also independent on the PGR5 and NDH-1 systems (Fig. 6A and B). In Physcomitrella, the P700 induction drastically differed from that of Arabidopsis by the strong FVL protein-dependent re-oxidation of P700 after the re-reduction phase (Supplemental Fig. 4), as recently reported with another moss species [45]. These results suggest that, both in the dark- and far-red-acclimated state, the saturating pulse first oxidizes the ETC, then the electrons from PSII reduce the chain, and finally, the chain is slightly re-oxidized probably due to the activation of PSI electron acceptor side in Arabidopsis and by the activation of FLV system in Physcomitrella.

Remarkably, since the P700 of *pgr5* behaved nearly similarly to that of WT after the 10-min darkness and 1-min far-red AL, the PGR5-dependent photosynthetic control seems to play only a minor role in the P700 oxidation in plants acclimated to darkness or far-red light. In contrast, the FLV proteins of Physcomitrella showed the strongest impact on P700 oxidation when the pulse was given after the darkness or far-red AL (Supplemental Fig. 4A and B) and, as described below, the difference between WT and *flvb* faded under high AL. Generally, the similar behavior of P700 in the dark and far-red conditions in Arabidopsis indicates that the far-red light illumination is insufficient to light-activate the chloroplast function, and instead, the regulatory mechanisms controlling ETC remain in inactive dark state even during far-red illumination. Conversely, the FLV system in Physcomitrella appears to be active already before the activation of photosynthesis.

When the saturating pulse was given after the 1-min low red AL, P700 was rapidly oxidized and remained oxidized during the whole pulse in Arabidopsis WT and *ndho*, but not in *pgr5* (Fig. 6C). This difference to the dark and far-red states indicates that the PGR5-dependent photosynthetic control requires pre-activation by light that drives photosynthetic electron transfer. Clearly, only when pre-activated by red light, PGR5 was capable of rapidly react to an increase in light intensity (Fig. 6C and D). After the 1-min high AL, the photosynthetic control was already activated and, thus, the saturating pulse failed to pointedly increase the P700 oxidation level in WT and *ndho* (Fig. 6D). In *pgr5*, on the contrary, P700 was largely reduced after the 1-min high AL illumination, yet the saturating pulse was still able to oxidize a small fraction of P700. This pulse-induced oxidation was, however, lost as a result of PSI photoinhibition, indicating that, in the absence of photosynthetic control, high light can oxidize P700 if the PSI to PSII ratio is high enough. When the saturating pulse was given after low AL, the Physcomitrella *flvb* differed from WT only in the end of the pulse (Supplemental Fig. 4C), but the difference was much smaller than after the acclimation to dark or far-red AL (Supplemental Fig. 4A and B). Moreover, after high AL, the difference between the mutant and WT nearly disappeared (Supplemental Fig. 4D). The results from the fluorescence and $P700^+$ induction in Physcomitrella suggest that the FLV proteins are needed to cope with high light pulses under a low-intensity and far-red-enriched canopy, whereas upon a prominent increase in light intensity, PGR5 system takes over.

3.4. The amount PGR5, NDH-1 and PTOX systems show a genotype-dependent response to PSI inhibition

The functional measurements (Figs. 3–5) indicated that both the PGR5 and the NDH-1 system play a role in the 24-h acclimation response of Arabidopsis after the PIT. Therefore, we were interested to see whether the acclimation to the PSI-limited state alters the amount of the PGR5 and NDH-1 systems. Because PTOX is known to be able to accept electrons from ETC instead of PSI, also the amount of PTOX was determined.

As demonstrated in Fig. 7, WT upregulated the level of PTOX and PGR5 as a response to the PIT and the subsequent 24-h recovery at growth conditions (Fig. 7). The *pgr5* mutant showed an elevated amount of PTOX already before PIT, but increased the amount of NDH-1, suggesting an improved capacity for chlororespiratory electron transfer pathway to protonate the lumen – a finding sufficient to explain why *pgr5* was able to keep P700 more oxidized under far-red light, as compared to WT (Y(ND) in Fig. 5A). Similarly to *pgr5*, also *ndho* upregulated the amount of PTOX already before PIT, but accumulated PGR5 protein as a response to PSI photoinhibition similar to WT and, thus, the increased photosynthetic control could explain the effect of PIT on the parameters Y(ND) and Y(NA) (Fig. 4A and B).

Residual amounts of PGR5 were seen in the western blot even in *pgr5*, due to the point mutation that still allows the translation of PGR5 although the mature protein is completely non-functional (Agrisera). Interestingly, opposite to WT and *ndho*, the amount of mutated PGR5 protein in *pgr5* decreased as a result of PIT, suggesting that the amount of PGR5 protein is dynamically regulated, probably according to the state of intersystem ETC or PSI electron acceptors. Moreover, the glycine-130 substituted by serine in the mutated PGR5 protein of *pgr5* not

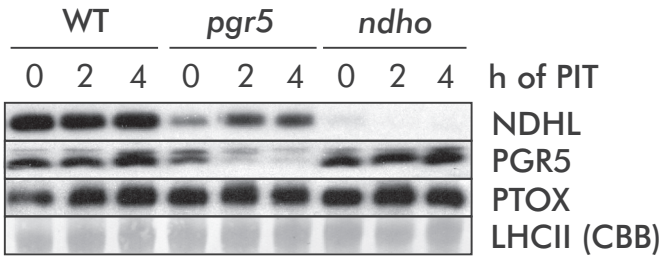

Fig. 7. Amount of NDHL, PGR5, and PTOX proteins 24 h after the PSI photoinhibition treatment. Thylakoid proteins isolated from leaves of *Arabidopsis thaliana* WT, *pgr5* and *ndho* after 0, 2 and 4 h of PSI photoinhibition treatment (PIT) were separated with SDS-PAGE, transferred to PVDF membrane and immunodetected with antibodies against NDHL, PGR5 and PTOX. Equal sample loading with Coomassie Brilliant Blue (CBB) staining. Before the experiments, the treated leaves were allowed to rebalance their photosynthetic machinery for 24 h under growth light conditions.

only prevents the function of the protein, but also seems to affect the molecular mechanism responsible for controlling the amount of PGR5 in the thylakoid membrane.

4. Discussion

Photosynthetic light reactions are dynamically regulated by changes in the light intensity but also by the capacity to collect available excitation energy, to convert it into chemical form and to utilize the chemical energy in metabolism. These changes are reflected in the pH of the thylakoid lumen and the redox state of the PQ pool, which both largely affect the molecular regulatory mechanisms controlling light harvesting and electron transfer reactions. The lumen pH modulates the thermal dissipation of excess excitation energy (NPQ) and the control of electron transfer at Cyt b_6f (photosynthetic control). The redox state of the ETC, on the other hand, controls the distribution of excitation energy between PSII and PSI (for reviews, see e.g. [27,46,47]), but is linked also to the regulation of lumen pH via the chloroplast thioredoxin network [48]. All these regulatory mechanisms eventually prevent PSI from accumulating excess electrons, which, if exceeding the capacity of electron acceptors, immediately inhibit PSI [1,20,31,49]. If the above-mentioned regulatory mechanisms fail to protect PSI, another strategy to avoid PSI inhibition is to provide alternative electron acceptors that can safely utilize the excess electrons [32].

According to the current knowledge, the PGR5–PGRL1 and NDH-1 systems are the only identified protein complexes able to function as alternative PSI electron acceptors in angiosperms and thus protect PSI from photoinhibition upon exposure to excess electrons (For recent reviews, see e.g. [8,50,51]. For this reason, we were interested to see, whether these mechanisms are able to protect PSI towards electrons accumulated in the ETC. Moreover, since the PGR5 and NDH-1 systems are known as the main mechanisms modulating the angiosperm electron transfer according to the environmental cues, we asked, if they are also capable of rebalancing the ETC after PSI photoinhibition and, consequently, of protecting the photosynthetic machinery during the slow process of PSI repair.

In order to test the possible roles of PGR5 and NDH-1 in preventing or mitigating PSI inhibition, we applied a recently developed light treatment for controlled PSI inhibition (PIT) [1] onto WT, *pgr5* and *ndho* (Fig. 2). The method is based on electron burst from over-reduced ETC to PSI, which, in WT, was shown to be able to induce various levels of PSI inhibition depending on the exposure time of PIT [1]. Recently, PSI has been inhibited also by high light treatment of *pgr5* [5] and by repetitive short saturating pulses under darkness [49]. Here, the method of PIT was chosen due to its applicability to different genotypes, including WT, and in order to create a situation, in which electrons have accumulated into ETC under light, when photosynthesis and the studied regulatory mechanisms also are active. PSI after the PIT is not able to recover during the following 24-h acclimation in the growth conditions [1] and, for this reason, we allowed the photoinhibited leaves to rebalance their photosynthetic machinery to the new PSI-limiting state for 24 h before any measurements.

Our experiments with the PSI-limited Arabidopsis WT, *pgr5* and *ndho* confirmed that PGR5 and NDH-1 do not function as protective electron acceptors, at least not after electrons have already accumulated in the ETC. Additionally, for the first time, the acclimation methods of Arabidopsis towards PSI photoinhibition are characterized by investigating the consequences of the inhibition on the electron transfer rate, PSI oxidation, relative Q_A reduction (Dual-PAM parameters) as well as on the amount of PSII, PSI, PGR5, NDH-1 and PTOX proteins (western blots).

4.1. PGR5 and NDH-1 systems do not function as protective electron acceptors

The PIT first over-reduces the ETC by illumination with low PSII-favoring i.e. red light and then allows PSI to drain the excess electrons from the ETC by applying a pulse of saturating white light [1]. The consequent burst of electrons saturates the capacity of Fd to accept electrons from PSI, which leads to damage of the FeS clusters and to immediate inhibition of PSI function (Fig. 2A and B). Theoretically, any alternative electron acceptor able to oxidize Fd, including PGR5 and NDH-1, is expected to decrease the acceptor side limitation of PSI and, thus, to protect PSI from photoinhibition. Undeniably, under fluctuating light, PGR5 becomes essential in protecting PSI against photodamage, and also NDH-1 is required under fast fluctuations [12,50]. Nevertheless, turnover of Fd has recently been shown to be independent of PGR5 or NDH-1 during steady-state photosynthesis [52,53], raising a question about the nature of the protection mechanism. Here, the response of *pgr5* and *ndho* mutants for the PIT was similar, yet slightly stronger, than that of WT (Fig. 2A and B). The slightly elevated sensitivity of *pgr5* can result from the mutant's inability to induce photosynthetic control during the pulse. This would allow the electrons accumulated during the 5-s PSII-exciting light to reach PSI during the subsequent pulse, causing the more prominent loss of PSI function. NHD-1, in turn, may increase the proton gradient across the thylakoid membrane during PSII-exciting light and thus, the *ndho* could suffer from a similar defect as *pgr5*, exposing its PSI to more electrons than WT. However, since the function of PSI drastically decreased as a response to the treatment not only in *pgr5* and *ndho* but also in WT, it is concluded that neither the PGR5 nor the NDH-1 system has the capacity to protect PSI upon a burst of excess electrons from PSI reaction center to Fd.

Unlike the PGR5 and NDH-1 systems, the FLV proteins, which are missing from angiosperms but present in other branches of the green lineage, have been clearly shown to be able to accept electrons from PSI and to donate them to oxygen in a reaction that protects PSI from photoinhibition [32,33]. Even when artificially expressed in Arabidopsis, the FLV proteins have been shown to be able to rescue the *pgr5* phenotype [54]. For this reason, we were interested to see the responses of Physcomitrella WT and *flvb* to the PIT. Intriguingly, WT Physcomitrella was nearly resistant against the PIT, whereas the *flvb* mutant behaved like Arabidopsis (Supplemental Fig. 1). Since PGR5 and NDH-1 are both conserved also in Physcomitrella [55,56], the ability of Physcomitrella WT to protect PSI against inhibition most probably derives exclusively from the electron acceptance capacity of FLV proteins. The result is well in line with the comprehensive comparison on the capacity of FLV to oxidize P700 in several plant species [45], confirming that FLV proteins – but not PGR5 and NDH-1 – are able to protect PSI upon a failure in proper control of electron transfer to PSI. Thus, in the absence of FLV proteins as electron scavengers, the only way for angiosperms to avoid the over-reduction of the PSI electron acceptors is to keep the ETC optimally oxidized in all possible light conditions by very precisely regulating the electron transfer from PSII to PSI. If, however, the mechanisms controlling the ETC fail, as is the case during the PIT, plants accumulate an over-reduced pool of Fd, which, instead of feeding FNR, can result in production of dangerous superoxide. Interestingly, the response of Y(NA) on PIT, however, revealed that, after the inhibition of one PSI, the acceptor side capacity of another PSI increases (decreased Y(NA)) in Arabidopsis but decreases in Physcomitrella *flvb* mutant (increased Y(NA)) (Fig. 5B and Supplemental Fig. 3B). It seems that after an inhibition of a fraction of PSI, Arabidopsis is able increase the capacity of the undamaged PSI centers, but Physcomitrella does not have such capacity. The result indicates that the loss of FLV proteins coincides with an elevated risk of PSI inhibition and, in order to minimize the effect of inhibition, angiosperms have evolved mechanisms to maximize the use of still active PSI centers.

The resilience of WT Physcomitrella to the PIT is well consistent with previous reports highlighting the essential role of FLV proteins in coping with fluctuating light regimes [57,58]. This ability most likely relies on high electron accepting capacity of the FLV-system that is

absent in the CET pathway. These FLV-dependent mechanisms were evident on the strong re-oxidation of P700 upon a saturating pulse on the Physcomitrella tissue acclimated to darkness and far-red AL (Supplemental Fig. 4A an B), whereas in Arabidopsis, such re-oxidation was completely missing (Fig. 6A and B). However, after acclimation to low AL the oxidation of P700 was equally fast in WT Physcomitrella and WT Arabidopsis (Fig. 6C and Supplemental Fig. 4C), demonstrating that rapid oxidation of P700 does not necessarily indicate that PSI is protected from photoinhibition.

The FLVA and FLVB of Physcomitrella show high similarity to the corresponding FLV1 and FLV3 isoforms of cyanobacteria, yet the exact electron donor for the Physcomitrella FLV proteins has not been confirmed [35]. Thus, the FLV proteins might dissipate the electron pressure to PSI via electron transfer to O_2 at different steps of the electron transfer downstream of PSI. They might accept electrons from NADPH [59], Fd [60] or even directly from PSI thus preventing the over-reduction of Fd pool. The effect of FLV proteins on P700 oxidation was visible only in the end of the saturating pulse, suggesting that the FLV system is activated rather slowly. This might indicate that the FLV proteins rather function in keeping the capacity of Fd and NADP/H systems high, instead of as direct electron acceptors of PSI. Only future experiments will clarify, which is the actual electron donor for FLV proteins and how the donation is regulated. Currently, we cannot exclude that specific regulatory mechanisms, e.g. redox regulation in the stroma [33], contribute to the prominent electron transfer via FLV in the first seconds after an abrupt increase in light illumination, while for longer light exposure, the role of FLV turns out to be negligible.

4.2. Activated PGR5 controls the electron flow at Cyt b_6f

Although the exact mechanism has remained elusive, PGR5 is known to keep PSI oxidized upon increase in light intensity in angiosperms [19–21,50,52]. Indeed, as demonstrated by the P700 kinetics, PSI of the light-acclimated WT and *ndho* plants was oxidized in milliseconds upon the saturating pulse, while the oxidation was missing from the *pgr5* leaves (Fig. 6C and D). Such a rapid oxidation of P700 was, however, not able to protect PSI from excess electrons that were already accumulated in the ETC prior to the saturating pulse and, as a consequence, also the PSI of WT and *ndho* was inhibited by the PIT. The result suggests that the PGR5 system rapidly and efficiently controls the electron transfer through Cyt b_6f, but in the case electrons have passed this regulatory step and already reduced PC, PGR5 is unable to protect PSI. Interestingly, despite the over-reduced ETC in low AL after PIT (Fig. 5C), the saturating pulse was able to oxidize P700 in WT and *ndho* (Fig. 6C). This indicates that P700 can become oxidized by over-reducing the stromal electron acceptors and thus the PSI-damaging reaction might occur despite the oxidation of P700. The distinctly different P700 oxidation kinetics of WT and *pgr5* leaves acclimated to the darkness (Fig. 6A and B) and to the light (figure C and D) clearly demonstrated that the PGR5 system also requires light-induced activation in order to be able to work upon sudden increase in the light intensity.

4.3. Rebalancing the redox state of ETC by thylakoid protein phosphorylation

PSI inhibition leads to limitation of photosynthetic electron transfer by altering the capacity of PSI and, consequently, results in over-reduction of the entire intersystem ETC (Fig. 5C). The accumulation of electrons in the ETC not only limits photosynthesis but also endangers the still functional PSI centers. In order to minimize the PSI limitation of electron transfer after PSI inhibition, the efficiency of the undamaged PSI centers has to be maximized. One way to accelerate the function of a PSI is to increase its relative antenna size by phosphorylating the LHCII proteins [29,31,61,62]: high LHCII phosphorylation [29] or rather high concomitant LHCII and PSII core protein phosphorylation [63] increase the relative excitation of PSI and thus helps PSI to balance the redox sate of ETC. Intriguingly, PSI inhibition by PIT increased the phosphorylation of LHCII and PSII core proteins in all genotypes (Fig. 3B). Earlier, the same phenomenon has been demonstrated by using high light treatment on *pgr5* [5,63], which is unable to control the electron transfer from PSII to PSI and which, consequently, suffers from PSI inhibition under high light [19]. In such high light stress conditions, PSI inhibition strongly increases the phosphorylation state of PSII−LHCII and, concomitantly, the relative excitation of PSI [5,63]. Based on these findings, Tiwari et al. (2016) suggested that the photoinhibited PSI can thermally dissipate the excitation energy and can thus function as an additional NPQ mechanism under excess light. Here, the plants were allowed to acclimate to the new energy-limited state 24 h after the PIT and, therefore, the wasteful energy transfer to the inhibited PSI centers should be rather avoided than preferred as in the case of high light. Nevertheless, both the phosphorylation of thylakoid proteins and the relative excitation of PSI were still seen pronounced (Fig. 3A). Despite the fact that the inhibited PSI can dissipate excess excitation energy, it is more likely that the increase in the relative excitation of PSI, as a result of PSI inhibition, rather enhances the light harvesting for the still functional PSI centers than increases the thermal dissipation capacity of the PSI-limited photosynthetic machinery. In fact, as described below, plants can compensate the decrease in the number of PSI by enhancing the function of the remaining PSI.

4.4. Mobile ETC components partly rebalance the ETC under light-limiting conditions

The comparison between the Dual-PAM parameters ETR(II) and ETR(I) revealed one mechanism accelerating the function of the undamaged PSI centers after PIT (Fig. 4). Intriguingly, the PIT strongly increased the electron transfer rate of PSI under light-limiting conditions and, at the same time, decreased that of PSII in all genotypes. In order to take a closer look at the phenomenon, the donor and acceptor side limitations of PSI were analyzed using the parameters Y(ND) and Y (NA), respectively (Fig. 5A and B). The PIT was found to decrease the donor side limitation of PSI therefore keeping PSI reduced under far-red and low AL, which both are incapable of inducing the PGR5-dependent photosynthetic control (Fig. 5A). This result indicates that the electron transfer from PSII to the still functional PSI is enhanced, inevitably suggesting that the PSII and PSI do not function as fixed pairs with designated ETC components, but rather as large pools that can flexibly utilize the common components of ETC. Thus, when the function of PSI starts to limit ETC, the reduced PC molecules are able to diffuse to functional PSI centers for oxidation, which accelerates the electron transfer through PSI (Fig. 4B). The concomitant low acceptor side limitation after the far-red and low AL demonstrates that the stromal acceptors are in excess under light-limiting conditions and, in fact, after the 4-h PIT, the acceptor side limitation completely disappeared in WT and *ndho*, and drastically decreased also in *pgr5*. The result demonstrates that, when the amount of functional PSI decreases, not only PC is able to find a functional PSI, but also the location of Fd and FNR is dynamically regulated according to the activity of the PSI centers. Such increase in the availability of electron acceptors in relation to the number of electron donors per functional PSI probably plays an important role in the protection of undamaged PSI. The increased availability of electron acceptors neutralizes the effect of PSI inhibition-induced accumulation of electrons in ETC that could otherwise result in a dangerous chain reaction eventually leading to a collapse of an entire PSI pool under the naturally fluctuating light conditions.

4.5. Increased amount and activity of PGR5 rebalances the ETC after PSI inhibition

While PSI photoinhibition leads to accelerated electron transfer through remaining functional PSI centers in all genotypes when measured under far-red and low AL, only WT and *ndho* showed similar

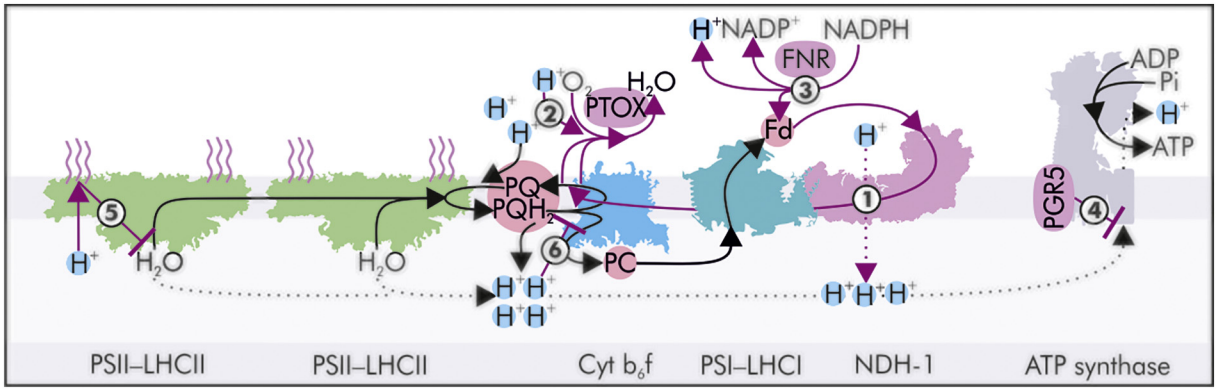

Fig. 8. Hypothetical scheme presenting angiosperm acclimation strategies to PSI-limited state. Plants increase the amounts of NDH-1, PTOX and PGR5 as a response to PSI photoinhibition. Under low and moderate intensities, the decreased amount of PSI limits electron transfer and thus chloroplast capacity to produce ATP and NADPH. The production of NADPH is dependent on PSI, but the production of ATP can be compensated by enhancing the generation of proton motive force by alternative mechanisms. (**1**) Electron transfer through NDH-1 is coupled to proton pumping from the chloroplast stroma into the thylakoid lumen and (**2**) PTOX utilizes protons in the stroma. (**3**) In addition, the FNR associated with inhibited PSI starts to function in reverse direction by oxidizing NADPH in order to produce reduced Fd for the chlororespiration. Under high light, (**4**) PGR5 controls the proton conductivity of the thylakoid membrane, possibly via ATP synthase conductivity, and (**5**) the consequently protonated lumen downregulates the function of PSII by NPQ and (**6**) limits the electron transfer to PSI by photosynthetic control at Cyt b_6f. Thus, only a small fraction of PSI is needed to provide sufficient electron transfer under high light. Moreover, the inhibition of PSI prevents the high light-induced dephosphorylation of LHCII proteins, which increases the antenna size of the still functional PSI (not shown in the figure). Membrane-bound PTOX and FNR are presented apart from the thylakoid membrane for clarity.

behavior also under high AL (Fig. 4B). In line with earlier reports [19–21], this PGR5 protein-dependent mechanism to keep PSI open under high light results from the enhanced donor side limitation of PSI, as demonstrated by the high Y(ND) parameter of WT in Fig. 5A. In the absence of the PGR5 protein, on the other hand, plants are unable to limit the electron transfer to PSI and this defect was seen as a low donor side Y(ND) and high acceptor side limitation Y(NA) of PSI after PIT (Fig. 5A and B). Such simultaneous reduction pressure on both the donor and the acceptor side exposes PSI to inhibition under any abrupt increase in light intensity. Interestingly, in WT and *ndho*, the inhibition of PSI resulted in an increase in the fraction of oxidized PSI centers when measured under high AL and, consequently, alleviated the acceptor side limitation (Fig. 5A and B), suggesting a limitation in electron flow from PSII to PSI. Actually, the high Y(ND) and low Y(NA) of WT and *ndho* were accompanied by an increased amount of the PGR5 protein (Fig. 7). This interesting result signifies that, in conditions endangering PSI, plants can enhance the function of PGR5 in order to limit the electron transfer to the still functional PSI centers and, consequently, to reduce the risk of further PSI inhibition. Moreover, the fact that the function of PGR5 is not dependent on the redox state of the PSI electron acceptors excludes PGR5 as a PSI electron acceptor and demonstrates that, instead, it modulates the electron transfer independently of the amount or activity of PSI.

We hypothesize that PGR5 slows down the ETC through downregulating ATP synthase (Fig. 7). This is based on the reports demonstrating that the *pgr5* mutant behaves oppositely to a *Nicotiana tabacum* mutant with decreased ATP synthase activity [66] and similarly to a mutant with increased ATP synthase activity [23]. In fact, *pgr5* shows a significantly higher proton conductivity of ATP synthase [18,67] and, vice versa, proton gradient uncoupler makes WT behave like *pgr5* [68]. As mentioned earlier, by controlling the proton conductivity of the thylakoid membrane, plants can control the electron transfer via Cyt b_6f as well as the thermal dissipation of excess excitation energy. The easiest solution to modulate the proton conductivity might be to control ATP synthase according the capacity of stromal components to utilize electrons and/or protons.

4.6. NDH-1 complex supports the lumen acidification in PSI photoinhibited chloroplasts

As demonstrated above, the inhibition of PSI limits LET and consequently also both the capacity of PSII to release protons in the thylakoid lumen as well as the capacity of Cyt b_6f to pump protons from the stroma into the lumen. However, in addition to PSI, the ETC can be oxidized by PTOX [16]. Such alternative oxidation pathway is expected to relieve the acceptor side limitation of PSII and to accelerate lumen acidification [69,70]. Since the PTOX-dependent oxidation of the PQ pool does not involve Cyt b_6f functioning as an electron transfer-coupled proton pump, the compensatory proton translocation from the stroma into the lumen may be supplied by NDH-1. Although the chlororespiration function of NDH-1 consumes NADPH from the reversely functioning FNR [71], during PSI-limited conditions it might be more important to produce ATP for protein synthesis, even at the expense of NADPH i.e. decreased carbon fixation. Therefore, as a response to PSI photoinhibition, plants increase the amount of both PTOX and NDH-1 (Fig. 7). Based on the fluorescence and P700 data presented here, it is hard to evaluate how significantly the increased amounts of PTOX and NDH-1 affect the chloroplast energy budget. It is also important to note that PGR5 is the dominant regulator of P700 oxidation and, thus, the effect of NDH-1 and PTOX might be largely masked by the function of PGR5, especially under high light. Yet, the capacity of *pgr5* to oxidize P700 was partially rescued after PIT (Fig. 4B and C), suggesting that the upregulated amount of NDH-1 and PTOX (Fig. 7) supports the protonation of lumen and enhances the photosynthetic control upon PSI limitation, seen as PGR5-independent limitation of LET. In addition to the photosynthetic control, such NDH-1- and PTOX-dependent chlororespiration might also be required to power the chloroplast metabolism upon PSI limitation by producing ATP.

The role of NHD-1 in rebalancing the ETC to the PSI-limited state might include accumulation of PSI–NDH-1 supercomplexes [10,11]. In fact, the *ndho* mutant upregulated the amount of PSI as a response to PIT (Fig. 1C), which might be an attempt to compensate for the absence of a functional PSI–NDH-1 supercomplex.

5. Conclusions

In the vast majority of the literature, the PGR5 and NDH-1 systems in Arabidopsis are described as CET pathways accepting electrons from PSI via Fd and are thus expected to alleviate the over-reduction of PSI acceptor side, thereby protecting PSI against photoinhibition. Nevertheless, we demonstrate here that neither PGR5 nor NDH-1 system is able to protect PSI from photoinhibition that is caused by a

sudden burst of electrons from PSI to Fd. This inevitably suggests that neither of these systems accepts electrons directly from the Fd that is reduced by the PSI receiving electrons from LET. Thus, we propose that the PSI–NDH-1 supercomplex mediates CET and balances the proton gradient at the nonappressed thylakoid membranes, separately from LET. In contrast, the FLV proteins in Physcomitrella are shown to efficiently protect PSI from the electron burst induced by our PIT, suggesting that they have high capacity to control the redox state of stroma and they might function as direct electron acceptors from PSI when the Fd pool becomes reduced. Instead of protecting PSI by accepting electrons, both the PGR5 and NDH-1 systems are here demonstrated to be upregulated by PSI photoinhibition and to protect the remaining PSI centers by enhancing the pH-dependent regulation of electron transfer from PSII to PSI (Fig. 8). Furthermore, it also seems likely that NDH-1 functions together with PTOX in order to increase ATP production upon PSI limitation (Fig. 8).

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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.

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